THESIS

BENZENE BIODEGRADATION IN COAL-TAR CONTAMINATED GROUNDWATER: DOCUMENTING THE ROLE OF *VARIOVORAX* MAK3

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BENZENE BIODEGRADATION IN COAL-TAR CONTAMINATED GROUNDWATER: DOCUMENTING THE ROLE OF *VARIOVORAX* MAK3

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Kevin M. Posman

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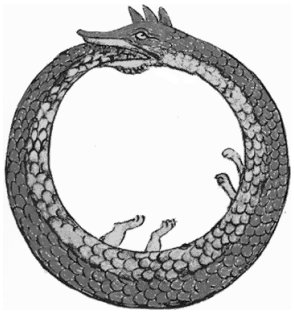
ABSTRACT

Microbial processes shape ecosystem functions and the fate of environmental Contaminants. Despite their importance little is known about the active players carrying out specific metabolic processes in the environment. Investigations of environmental microbial communities are crucial for understanding the diversity of microorganisms capable of degrading these compounds and will provide insight into bioremediation strategies. The aim of this study was to use cultivation-dependent and cultivation-independent techniques to examine benzene-degrading microorganisms in hydrocarbon-contaminated groundwater. We investigated groundwater samples from a coal-tar contaminated aquifer in South Glens Falls, NY undergoing monitored natural attenuation (MNA). Previous stable isotope probing (SIP) experiments using ^{13}C-labelled benzene in groundwater microcosms from the site revealed a high abundance of γ- and β-proteobacteria were active in the biodegradation of benzene. To further explore the microbiology of this system, we isolated benzene-degrading microorganisms from the SIP microcosm experiments. Nine organisms were isolated and identified using 16S rRNA gene sequences and several shared >97% nucleotide identity to 16S rRNA gene sequences retrieved in the SIP experiments. Isolate *Variovorax* MAK3 was chosen for further characterization. We designed primers to investigate a putative ring-hydroxylating dioxygenase (RHD) hypothesized to be involved in its ability to degrade benzene. We demonstrated that *Variovorax* MAK3 accelerated benzene degradation microcosms prepared from site waters and quantified a corresponding increase in *Variovorax* RHD gene expression over the same time period. Finally, we show that when the native community was exposed to benzene the ratio of RDH dioxygenase to 16S rRNA gene transcripts of native *Variovorax* populations increased over 6 fold during the benzene biodegradation. These data demonstrate how the convergence of cultivation-dependent

and cultivation-independent techniques can lead to a precise understanding of active populations and their biodegradation genes in complex microbial communities.

BIOGRAPHICAL SKETCH

Kevin Michael Posman was born in Albany, New York and grew up in Rochester, NY. He lucked out in the birth lottery and was raised by loving parents parents Ken and Ann and was eventually joined by his sister Mary. He studied science and philosophy at the Rochester Institute of Technology and graduated with a BS in Biotechnology in 2008. His undergraduate thesis examined the link between exposure to animal waste and increases in antibiotic resistance among enteric bacteria of wild leopard frogs. After a brief summer working at an industrial biotechnology company an interest in microbiology led him to pursue graduate training at Cornell University in the fall 2008. He lucked out again while studying in Ithaca by meeting Julia Brown and falling in love.



DEDICATION

To all of us

"Perhaps we will continue believe in change and real democracy.
Yet democracy, a system to challenge the status quo has been corrupted to serve the status quo. We will be left with nowhere to turn and instead choose inaction.
The idea that we can always make more money, meet new quotas, consume more products [write more grants, publish more papers], and advance our careers, this idea that human and personal progress is some how inevitable leads to political passivity.
Our passivity is due in part to our inability to confront the awful fact of extinction, either our own inevitable morality or that of the human species. The emotional cost of confronting death is painful and we prefer illusion."

- Chris Hedges, *Death of the Liberal Class*

"We notice that everywhere, together with freedom, justice is profaned.
Perhaps we cannot prevent this world from being a world in which children are tortured.
But we can reduce the number of tortured children.
It is better to suffer certain injustices than to commit them, even to win wars.
Don't walk in front of me, I may not follow. Don't walk behind me, I may not lead. Walk beside me and be my friend. I shall tell you a great secret, my friend.
Do not wait for the last judgment, it takes place every day."

- Albert Camus, *Resistance, Rebellion and Death*

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Eugene Madsen, for his patience, guidance and for sharing his insights into all things from the ‘big picture’ to the microscopic. I would also like to thank my minor advisor, Dr. Ian Hewson for his advice and support throughout this process. I must also thank other Madsen lab members past and present: Jane Yagi, Buck Hanson, Chris DeRito, and Stuti Fernandes for good conversations and for being all-around great human beings. Thanks to Shirley Cramer for all she does and all the wonderful people of Wing Hall. Life in Ithaca would not have been the same without all the great people, you know who you are. My friends and family for all their love and support Mary, Ann, Ken (MAK). And finally big, big thanks to my great love and sojourner in life Julia M. Brown.

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CHAPTER ONE

Introduction

1.1 Benzene as an environmental pollutant

Benzene is the simplest aromatic hydrocarbon (C_6H_6), a known carcinogen and an environmental pollutant (Andreoni and Gianfreda 2007). Benzene is a highly volatile and relatively water-soluble, colorless liquid, which makes accidental exposure via inhalation or ingestion, a serious health concern (Table 1.1) (Harwood and Gibson 1997, Casarett and Doull 1996, ASTDR 2007). The widespread use and manufacture of benzene has led to hazardous occupational and non-occupational exposures and created broader environmental impacts (Andre oni and Gianfreda 2007). The toxic effects of benzene exposure were noticed early in occupational settings and the first cases of chronic benzene hematoxicity were documented 1897 (Smith 2010). Benzene toxicity affects the liver and chronic exposure to benzene leads to bone marrow damage, which causes, anemia, leukopenia, or thrombocytopenia (Casarett and Doull 1996). Non-occupational exposure is most commonly from cigarette smoking, combustion exhaust or proximity to point sources such as petrochemical plants and gasoline contaminant groundwater (Wallace 1996, Smith 2010).

There are a variety of natural and anthropogenic sources of benzene. Natural sources are predominately the gaseous emissions of volcanoes and forest fires (ASTDR 2007). However, the anthropogenic production of benzene by chemical manufacture and petroleum refinement is mainly responsible for the increase of benzene in the environment (Andreoni and Gianfreda 2007). Benzene is one of the top 20 chemicals produced by volume in the United States and worldwide (ATSDR 2007, Casarett and Doull 1996). Prior to World War II benzene was

Characteristic	Information	Reference
Chemical name	Benzene	HSDB 2007
Synonym(s)	Annulene, benzeen (Dutch), benzen (Polish), benzol, benzole; benzolo (Italian), coal naphtha, cyclohexatriene, fenzen (Czech), phene, phenyl hydride, pyrobenzol, pyrobenzole	HSDB 2007
Registered trade name(s)	Polystream	IARC 1982
Chemical formula	C_6H_6	Budavari et al. 2001
Chemical structure		Budavari et al. 2001
Identification numbers:		
CAS registry	71-43-2	HSDB 2007
NIOSH RTECS	CY-1400000	HSDB 2007
EPA hazardous waste	NA	
OHM/TADS	No Data	
DOT/UN/NA/IMCO shipping	UN1114; IMO3.2	HSDB 2007
HSDB	35	HSDB 2007
NCI	C55276	HSDB 2007
Merck	1066	Budavari et al. 2001

CAS = Chemical Abstracts Service; DOT/UN/NA/IMO=Department of Transportation/United Nations/North America/ Intergovernmental Maritime Dangerous Goods Code; EPA = Environmental Protection Agency; HSDB=Hazardous Substances Data Bank; NCI = National Cancer Institute; NIOSH= National Institute for Occupational Safety and Health; OHM/TADS = Oil and Hazardous Materials/Technical Assistance Data System; RTECS = Registry of Toxic Effects of Chemical Substances

Table 1.1 Chemical Identity of Benzene (ASTDR 2007)

refined from the light oil fraction produced during the coking process of coal tar (IARC 1982). Currently, greater than 98% of benzene produced in the United States originates from the petrochemical and petroleum industries; predominately by catalytic reforming and the production volume is estimated to rise (OSHA 1987, Greek 1990, ATSDR 2007). It is estimated that the global demand for benzene will increase by 6 million tons and value over $52 billion dollars by 2018 (Ceresana 2011).

Benzene is part of a pervasive family of monoaromatic hydrocarbon pollutants known as BTEX, which includes benzene, toluene, ethylbenzenes, and xylene that frequently co-occur at contaminated sites (Andreoni and Gianfreda 2007). Benzene and BTEX compounds are a widespread problem in groundwater due to discharge of industrial effluents, leaks from storage lagoons and underground containers, and spills from petroleum production, refinement and distribution (Fries et al 1994). Benzene represents 0.6% to 1% of gasoline and BTEX compounds collectively comprise about 15% of gasoline by volume (Harwood and Gibson 1997, Smith 2010). It is estimated that as many as 35% of the approximately 1.4 million tanks in the United States are leaking (Harwood and Gibson 1997). Moreover, benzene has been found in nearly 60% (1,000 of the 1,684) of the National Priority List (Superfund) sites identified by the Environmental Protection Agency (EPA) (Figure 1.1) (HazDat 2006). The continued mass production of benzene makes benzene pollution a serious and ongoing public health concern and a priority for remediation at impacted sites.

1.2 Benzene pathways, genes, model organisms

Benzene is a historically important molecule in organic chemistry as the central structure of the aromatic hydrocarbon family and was one of the first chemical constituents to be isolated

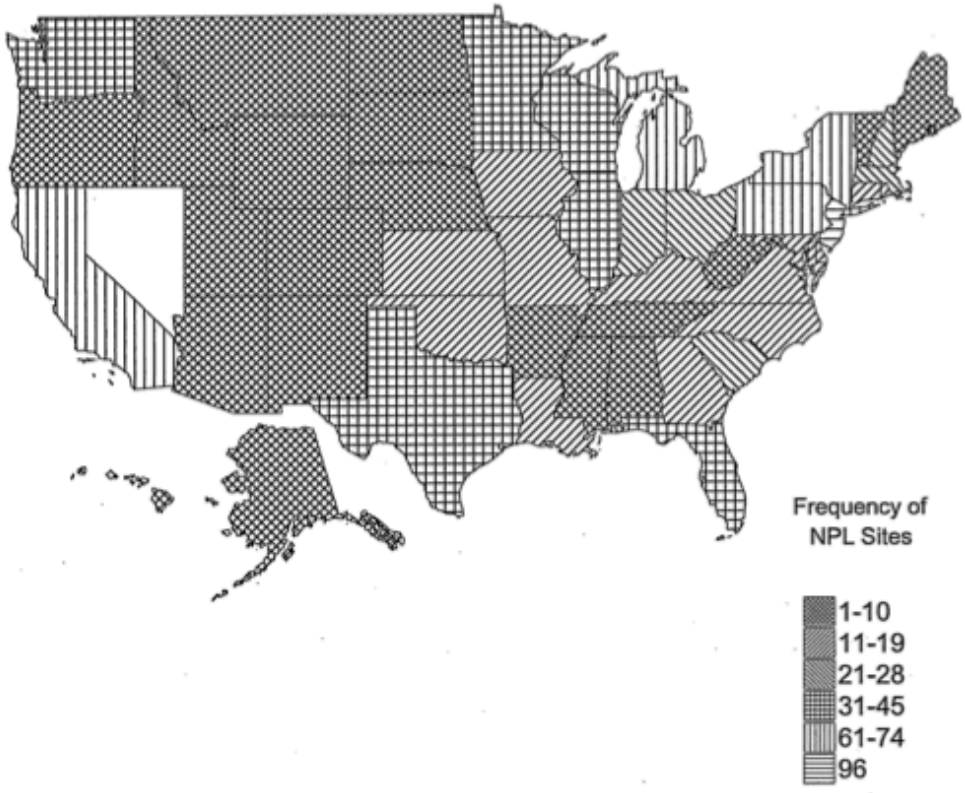

Figure 1.1 Frequency of National Priority List Sites with Benzene Contamination

from oil and coal tar (Faraday 1825, Hofmann 1845, Hoffman 1856, Kekulé 1866). Benzene is a model organic compound and one of the first reported hydrocarbons as a substrate for microbial aerobic and anaerobic conditions (Gibson and Parales 2000, Gibson and Harwood 2002). The aerobic metabolism of benzene begins with the incorporation of oxygen into the aromatic ring, either by monooxygenase or a dioxygenase attack (Andreoni and Giafreda 2007, Johnson and Olsen 1995). Monooxygenase incorporates an oxygen atom into the benzene ring and forms a phenol that is then converted by another monoxygenation into a catechol (Figure 1.2) (Tao et al 2004, Cao et al 2009). In contrast, the dioxygenase attack on the benzene incorporates two oxygen atoms into the benzene ring and produces a distinct benzene *cis*-dihydrodiol intermediate (Gibson et al 1970). Both pathways converge and form catechol that is cleaved via ortho- or-meta ring fission and continue to the tricarboxylic acid cycle to generate cellular energy and biomass (Figure 1.2) (Andreoni and Giafreda 2007).

The *bed* gene cluster *P. putida* ML2 is one of the most extensively studied dioxygenase systems (Butler and Mason 1997, Link et al 1996, Mason et al 1997, Bagneris et al 2005). The catabolic plasmid pHMT112 contains genes encoding the benzene dioxygenase (*bedC1C2BA*) as well as NAD^+-dependent dehydrogenase (*bedD*), which convert the *cis*-dihydrodiol to catechol (Tan and Mason 1990, Fong et al 1996). The benzene dioxygenase (EC 1.14.12.3) is comprised of a 3 component enzyme system, consisting of a oxidoreductase (*bedA),* which accepts electrons from NADH and transfers them to a ferrodoxin (*bedB*), which shuttles them to the terminal oxygenase, a 2 subunit Iron-sulfur protein (α-*bedC1 and* β-*BedC2*), which catalyzes the oxidation of benzene to *cis*-dihrodiol (Figure 1.3) (Axcell and Geary 1975, Tan and Mason 1990) . The *bed* iron-sulfur protein contains a Rieske-type cluster and a mononuclear non-heme iron oxygen activation center, which places it among large family of aromatic-ring-hydroxylating

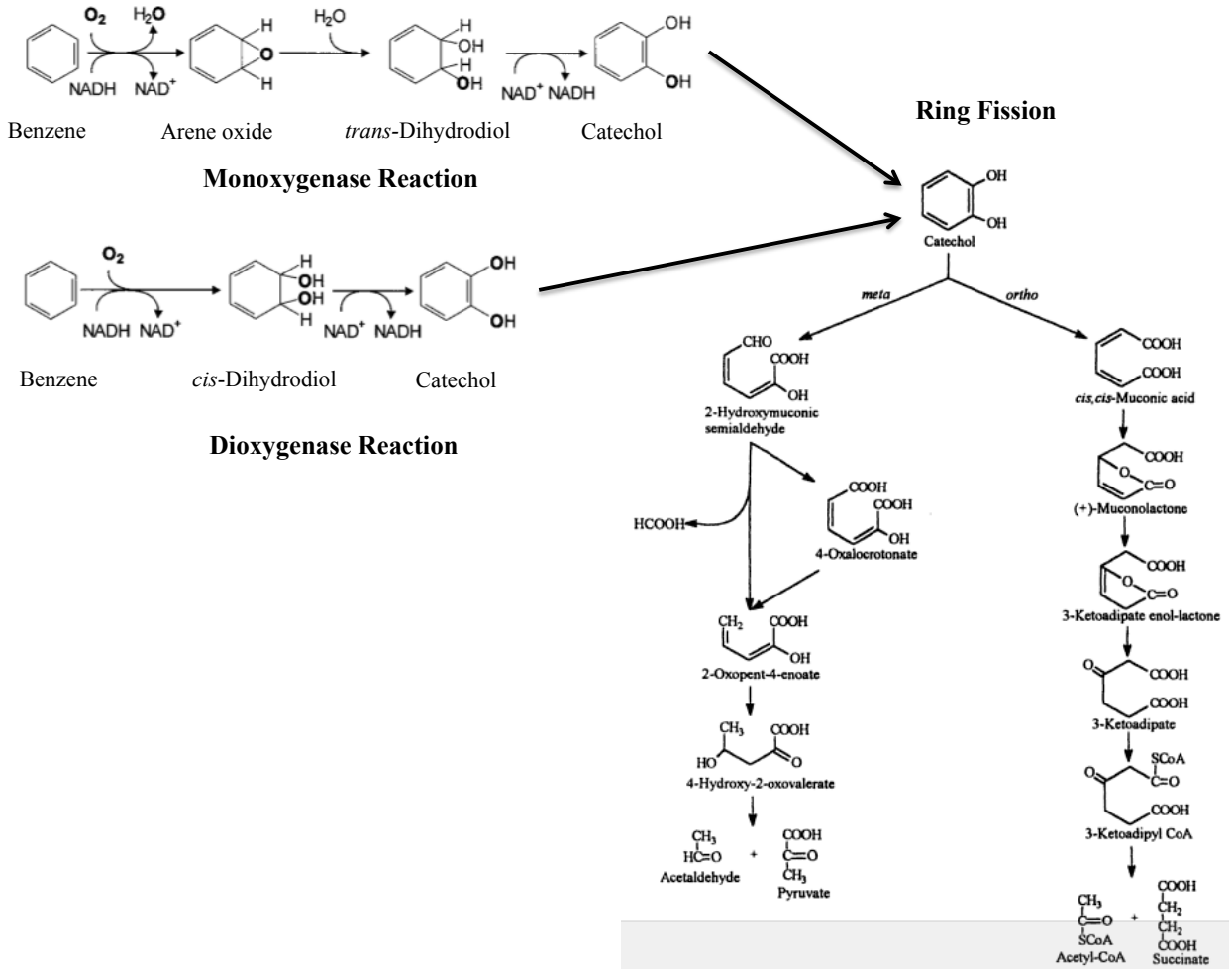

Figure 1.2 Dioxygenase, monooxygenase and catechol pathways of benzene metabolism (Fritsche and Hofrichter 2008, Agteren at al 1998).

dioxygenases (Bagneris et al 2005, Gibson and Parales 2000). The α-subunit is responsible for the difference in substrate specificity and differs by 33 amino acids from toluene dioxygenase (EC 1.14.12.11), which degrades toluene and ethylbenzene (Bagneris et al 2005). The α-subunits of benzene, toluene, and chlorobenzene dioxygenases are closely related and together they represent the D.2.C family of dioxygenase enzymes (Baldwin et al 2003).

Aerobic benzene degradation has been observed in several genera of Gram-positive and Gram-negative bacteria including: *Psuedomonas, Microbacterium, Azoarcus, Rhodococcus*, *Mycobacterium,* and *Bradyrhizobium* and the fungus *P.chrysosporium* (Andreoni and Gianfreda 2007, Yadav and Reddy 1993). More recently anaerobic benzene degradation has been observed and while aerobic degradation was elucidated over 50 years ago, little is known about the anaerobic pathways (Gibson and Harwood 2002, Chakraborty and Coates 2004, Vogt et al 2011).

The initial steps of anaerobic benzene degradation are hypothesized to include carboxylation, hydroxylation, methylation, or reduction of the aromatic ring to form benzoyl-Co A (Chakraborty and Coates 2004, Andreoni and Gianfreda 2007). Carbon dioxide, sulfate, iron (III) oxide, nitrate, chlorate have all been proposed as electron acceptors, yet only four anaerobic benzene degrading pure cultures have been described and they all require nitrate as the electron acceptor (Vogt et al 2011). There is evidence of degradation under some of these conditions in the field and that syntrophic consortia may paly a role in degradation, but cultivation remains a challenge in isolating these community members (Vogt et al 2011).

Decades of research on microbial benzene have identified and characterized a large diversity of benzene-degrading organisms and genes yet the full diversity of organism remains unexplored (Cavalca et al 2004, Gülensoy and Alvarez 1999, Gibson and Parales 2000). More remains to be done investigating the microbial ecology of BTEX bioremediation (Baldwin et al

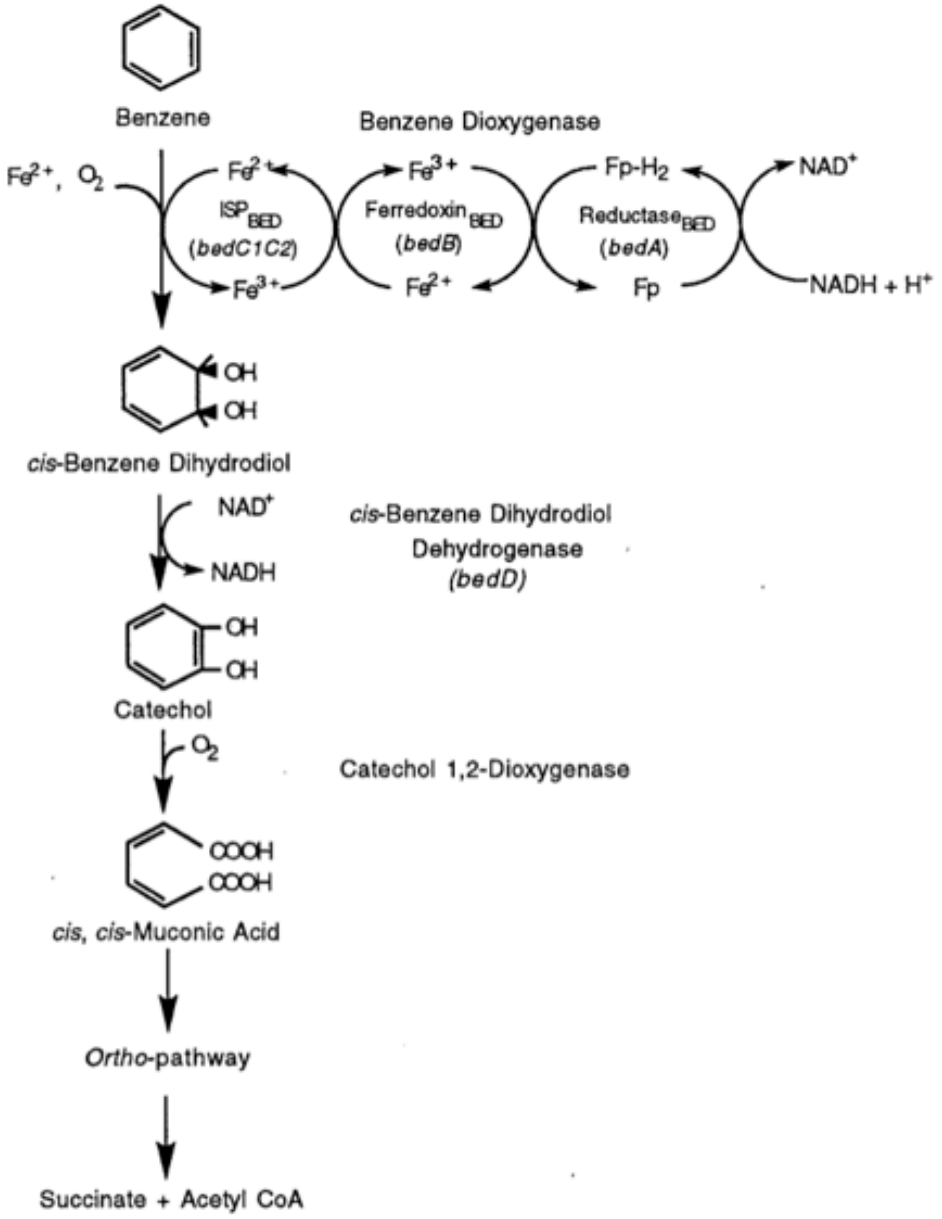

Figure 1.3 Initial reactions and enzyme components of the dioxygenase involved in the metabolism of benzene by *P. putida* ML2 (Fong et al 1996).

2003). Future investigations could reveal new organisms that can remove toxic substances, increase the toolbox for assessing *in situ* bioremediation potential and activity, discover new sources of genetic material for engineering, and novel mechanisms for chemical synthesis (Gibson and Parales 2000, Van hamme et al 2003).

1.3 Microbial ecology: linking identity with function

Microbial communities play a significant role facilitating nutrient cycling in Earth's ecosystems (Schlesinger 1997, Madsen 2011). They also act as master recyclers by mineralizing, transforming or immobilizing environmental pollutants and are capable of remediating contaminated sites (Diaz 2004). Despite their importance little is known about the vast taxonomic and functional genetic diversity of these organisms and their role as agents of geochemical change (Madsen 1998, Rappé and Giovannoni 2003). Achieving a more robust understanding of the key players and processes is constrained by the complexity of environmental communities and methodological limitations, particularly with traditional cultivation-dependent approaches (Madsen 1998, Madsen 2005). Environmental microbiology presents unique challenges for cultivation strategies because of the multifarious growth requirements and complex environmental matrices (Madsen 1998). This approach requires the perturbation of native habitat and selects for specific physiological traits that artificially alters the microbial community (Amann et al 1995, Madsen 1998, Paerl and Steppe 2003).

Molecular biology has created new means to identify taxonomic and functional genetic markers and revealed the inadequacy of culture-based methods to measure diversity (Rappé and Giovannoni 2003, Schloss and Handelsman 2004). It is estimated that >99% of microorganism remain uncultivated and half of the 52 bacterial phyla are represented by 16S rRNA sequences

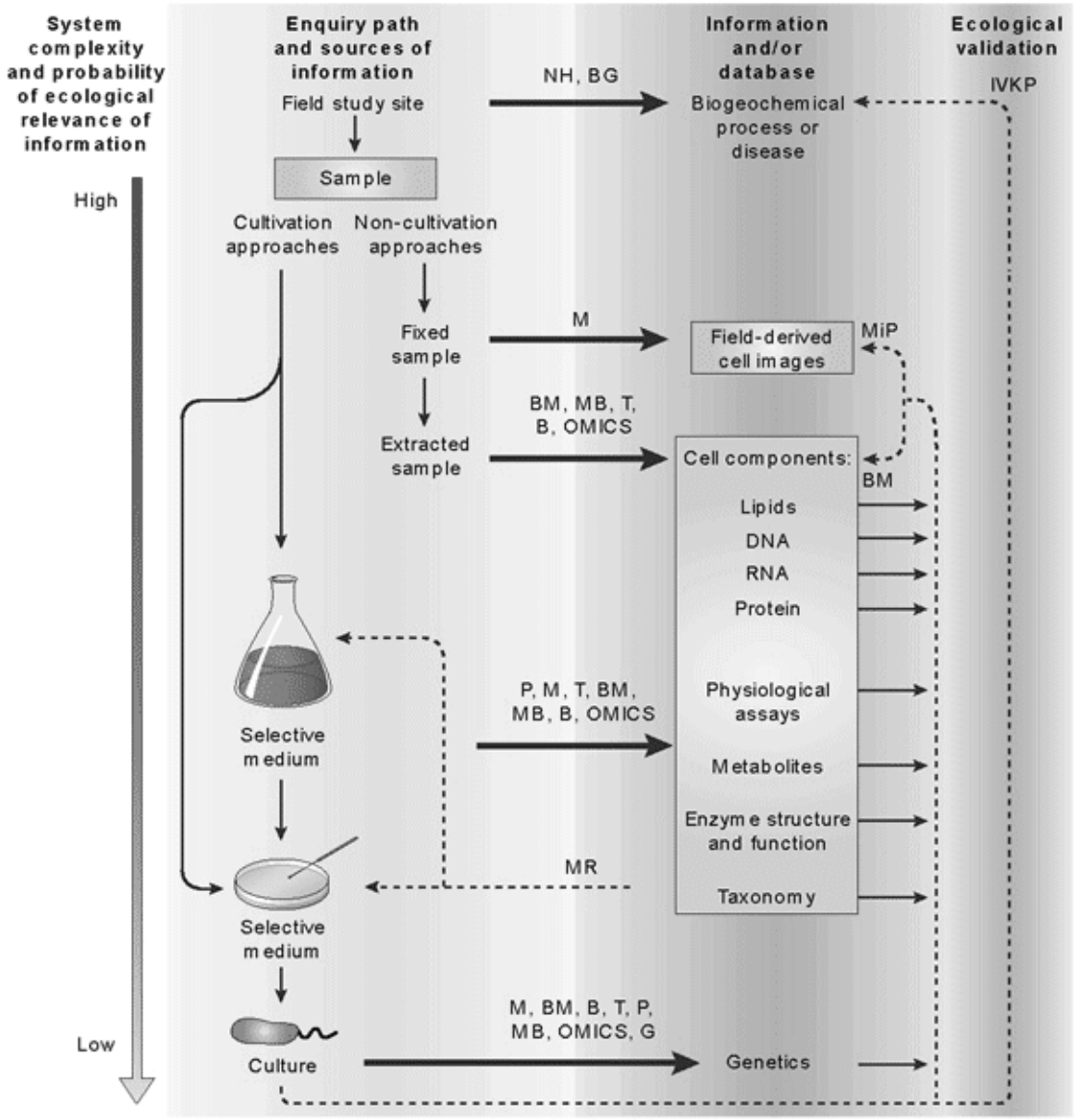

Figure 1.4 Model for the generation and interpretation of environmental microbiological information with emphasis on the field relevance and ecological validation of data (Madsen 2005).

of uncultivated microorganisms (Friedrich 2006, Schloss and Handelsman 2004). Increasingly, new techniques are being utilized to circumvent cultivation by directly extracting and analyzing biomarkers from the environmental, including DNA, RNA, protein and metabolites (Miller et al 1999, Wilson et al 1996, Wilson et al 1999, Wilmes and Bond 2004).

Polymerase chain reaction (PCR)-based investigation of DNA are a highly specific and se nsitive way of detect taxonomic or functional biomarkers in a complex community. However, separately taxonomic and functional gene information is insufficient to resolve 'who' is carrying out 'what' process and reveals nothing of the physiological status of the community. Quantifying the abundance of a particular sequence using quantitative PCR (qPCR) allows correlation between abundance and activity (Baldwin 2003). Quantification of mRNA (short-half life) provides insight into the functional genes being transcribed in response to current environmental stimuli (Smith and Osborn 2008). Similarly, isolation of proteins and metabolites provide a snapshot of the current metabolic process, but the challenges remain to link the process to a specific organism or gene (Wilmes and Bond 2006, Wilson et al 1996). Moreover, these approaches are limited to the current database of known taxa and functional genes, protein families and biochemical pathways.

The emergence of high throughput (HTP) sequencing, and analytical chemistry combined with computational bioinformatics has led the 'omics' era that makes community wide analyses for these biomarkers possible (Ryan and Robards 2006, Suenaga 2012, Poretsky et al 2009, Keller and Hettich 2009). Meta-omic techniques allow for the production of incredible amounts of information without *a priori* knowledge of community biomarkers. However, the tremendous increase in information has created a crisis of data analysis (Kowalchuk et al 2007). The bioinformatics challenge is to piece together info about the important populations and functions

(Kowalchuk et al 2007 and Suenaga 2012).

Stable isotope probing (SIP) is a technique that links identity to function by tracing the degradation of a particular substrate to the responsible community (Wackett 2004). The isotopically labeled substrate is physically incorporated into the biomass the microorganisms that are the active metabolic assimilation the compounds (Radajewski et al 2000). Stable Isotope probing (SIP) can be paired with the meta-omic approach of extracting and isolating the labeled biomass and has been applied to DNA, RNA, PLFA, protein and metabolites (Radajewski et al 2000, Whiteley 2006, Boschker et al 1998, Jehmlich et al 2010, Mosier 2013). SIP is minimally invasive and has been used in the field to discover organisms responsible for *in situ* metabolism and trace the path of compounds through microbial food webs (Jeon et al 2003, DeRito et al 2005). SIP is not without pitfalls, investigations are limited by a small selection of commercially produced substrates, and inadequate labeling, high GC content or carbon cross feeding can contribute to improperly separated biomarkers (Neufeld et al 2007, Uhlik 2013). A combination approach of both cultivation and cultivation- independent investigations has been shown to generate novel information about the roles of individual microbes within microbial communities (Figure 1.4) (Madsen 2005).

1.4 Microbial metabolism of environment pollutants

Anthropogenic release of hydrocarbon compounds into the environment has greatly impacted natural ecosystems (Reid et al 2000). Microbial communities are capable of responding to these perturbations and remediate-contaminated sites (Yagi et al 2009, Yagi et al 2010). Once in the environment the fate of benzene is governed by soil type and site geochemistry, principally the presence of oxygen (Andreoni and Gianfreda 2007). Microbial mediated

biodegradation often corresponds to decrease oxygen concentration and the accumulation of reduced chemical species (National Research Council 2000).

Numerous investigations have been launched to elucidate the impacts of hydrocarbon population on native communities (Leahy et al 1990, Head et al 2006). Studies have revealed that large inputs of pollution reduce diversity of microbial communities in the impacted environment (Roling et al 2002). Less abundant community members in pristine sites can grow to be the majority the community after exposure to organic pollutants (Andreoni and Gianfreda 2007). Community selection is not solely a function of the type of hydrocarbon pollution, Juck et al show geographic origin was a stronger selector and that different sites that share similar hydrocarbon-perturbations can have unique populations (Juck et al 2000). As bioremediation activity progresses in a given site, the hydrocarbon degrading communities undergo shifts not only in composition but catabolic gene expression (Vinas et al 2 005, Yagi et al 2009). The new metabolism promote new communities, Fahy et al (2005) examined the impacts of benzene pollution on microbial community diversity and showed that the metabolism of benzene was the key impact on community structure rather than direct chemic al toxicity. Indeed, several studies have shown hydrocarbon contaminated sites develop distinct microbial comminutes (Madsen et al 1991, Yagi et al 2010). In addition to understanding the dy namics of community composition more needs to be done to identify the active players responsib le for hydrocarbon consumption.

Aerobic benzene degrading bacteria have been isolated and cultivated from habitats that include soil, marine waters, and deep sea sediments (e.g. *Pseudomonas* (Arenhgi et al 2001), *Rhodococcus* (Kim et al 2002, Taki 2007) *Marinobacter* (Nicholson and Fathepure 2005), *Ancinetobacter* (Wang and Shao 2006), *Exiguobacterium* and *Bacillus* (Wang et al 2008).

PCR-based approaches have detected aerobic benzene degrading bacteria in many more habitats including sewage, hypersaline soil, deep ocean and groundwater and in far greater diversity (Cavalca et al 2004, Nicholson and Fathepure 2005, Hendrickx et al 2006, Wang et al 2008, Redmond and Valentine 2012). There has been an extensive accumulation of inquires in the genetic divert of oxygenase gene in naturally occurring microbial communities that maybe involved in BTEX Degradation (Baldwin et al 2003, Witzig et al 2006, Hendrickx et al 2006, Hendrickx et al 2006, Iwai et al 2011). Hendrickx (2005) used in situ mesocom system to measure the catabolic and community response to introduction of BTEX contamination and demonstrated a rapid shift to a stable community distinct from the uncontaminated treatment.

Quantitative PCR approaches have also been used to assess metabolic activity in subsurface habitats. Nebe et al 2009 and Baldwin et al 2010 used qPCR to evaluate the community response to the introduction of oxygen-releasing materials (ORMs) at a BTEX contaminated site. Both these investigations successfully linked increased oxygen concentration to increase aromatic oxygenase gene transcription and increased in 16S rRNA copies of specific organisms. Yagi et al 2009 and Tancsics et al 2012 demonstrated the native expression of dioxygenase genes in relation to the natural variations of groundwater geochemistry.

Stable isotope probing has been use to merge function and taxonomic identified of benzene degrading communities. In 2008, Liou et al compared aerobic benzene metabolizing communities from laboratory incubations to communities *in situ* using stable isotope probing. SIP identified new benzene degrading organisms that resist cultivation and for which there are no molecular probe (Xie et al 2011).

Linking microbial community structure with function is of particular interest in studying the metabolism of environmental pollutants. The long-standing goal remains to understand what

populations are responsible for the degradation of specific compounds in complex naturally occurring microbial communities(Madsen 2006). The work reported in the present thesis makes progress in this area. The microbial community investigated was groundwater from a contaminated field site in South Glens, Falls, NY. Our model system was freshly gathered groundwater incubated in the laboratory. We applied physiological-, stable isotope probing-, and cultivation-basedprocedures to identify bacteria and their biodegrative genes that carry out benzene biodegradation in the groundwater microbial community.

CHAPTER TWO

Benzene Biodegradation in Coal-tar Contaminated Groundwater: Documenting the Role of *Variovorax* MAK3

2.1 Introduction

The widespread use and manufacture of benzene has led to hazardous occupational and non- occupational exposures and created broader environmental impacts (Andreoni and Gianfred a 2007). Moreover, benzene has been found in nearly 60% (1,000 of the 1,684) of the National Priority List (Superfund) sites identified by the Environmental Protection Agency (EPA) (Figure 1.1) (HazDat 2006). The continued mass production of benzene makes benzene pollution a serious and ongoing public health concern and a priority for remediation at impacted sites (ASTDR 2007).

Microbial communities play a significant role facilitating nutrient cycling in Earth's ecoystems (Schlesinger 1997, Madsen 2011). They also act as master recyclers by mineralizing, transforming or immobilizing environmental pollutants and are capable of remediating contaminated sites (Diaz 2004). Despite their importance little is known about the vast taxonomic and functional genetic diversity of these organisms and their role as agents of geochemical change (Madsen 1998, Rappé and Giovannoni 2003).

This study aim to use cultivation dependent and cultivation-independent techniques to identify benzene-degrading organism in coal-tar contaminated water. This multifaceted approach allowed us to identify 'active' community members in benzene degradation and produced new information on functional genes responsible for this process.

2.2 Material and Methods

Site Description and Sample Collection

The study site, site 24, is a coal-tar contaminated aquifer located in South Glens Falls, New York. In the 1960s, coal-tar waste was deposited in an unlined pit at site 24 and groundwater carried leachate into the aquifer (Murarka et al 1992). The source material was removed in 1991, but a contaminated plume containing polycyclic hydrocarbons and other monocyclic constituents remained down gradient of the original source. Long-term monitoring of the contaminate plume has produced a robust record of groundwater chemistry and extensive documentation of contaminate-degrading microorganisms and made site 24 a model study site for of natural attenuation of aromatic hydrocarbon pollution.

Samples were taken from two geochemically distinct wells, well 36 and well 12. Well 36 is characterized by high levels of contaminants and anoxic water. Conversely, Well 12 is located further down gradient and is the least contained monitored well. These profiles generally true however, some geochemical data for site 24 indicate contaminant and oxygen concentrations in the plume experiences fluctuations and suggests the groundwater system is dynamic (Neuhauser et al 2009, Yagi et al 2010). Groundwater was sampled from the monitoring wells as per Bakermans et al 2002, transported on ice and stored at 4°C until the incubations.

Benzene degradation microcosm incubations

Benzene degradation by well water microbial communities

Cultures were grown from freezer stocks on BSM plates at 21°C for 7 days. Single colonies were picked and inoculated into 5mL BSM broth in a Balch tubes. For initial screening of benzene-degradation activity, liquid benzene was added to BSM media in unreplicated tubes to a concentration of 3 ppm, which were crimp sealed with Teflon-butyl rubber stopper. Cultures

were incubated at 21°C shaking at 120 rpm. The control was autoclaved sterilized *P. putida* F1. The second, more rigourous degradation experiment was conducted in triplicate, the cultures were grown on R2A, pelleted by centrifugation at 7000g, and washed in BSM (3x) and added at an initial concentration 6 x 10^6 cfu/mL to 80mL sterile BSM in serum bottles. Liquid benzene was added to the culture approximately at approximately 12 ppm. The cultures were shaken at 120 rpm at and Incubated at 21°C. Abiotic control treatments received no inoculum. Headspace gases were supplied periodically and measured for benzene concentration.

Dioxygenase Expression in minimal media

Cells were grown in BSM containing 0.5 g/L glucose, washed as described above and added to 80 mL BSM liquid media in serum bottles for final concentration of approximately 6 x 10^6 CFU/mL. Liquid benzene was added to the culture approximately at approximately 12 ppm. The two control treatments included one with *Variovorax* Strain MAK3 cells killed with 5M hydrochloric acid and the other did not receive inoculum. Cultures were incubated at 21°C and not shaken.

Dioxygenase expression in nutrient-amended well water

For the final degradation experiment 80mL of well 36 water amended with filter-sterilized $(NH_4)_3PO_4$ (final concentration of 10 mM) was added to serum bottles. *Variovorax* Strain MAK3 cells were grown as above and added at an initial concentration 2 x 10^5 cfu/mL. Liquid benzene was added to the culture at approximately 1.2 ppm. Controls were killed with 5M Hydrochloric acid. Cultures were incubated at 21°C and not shaken.

Isolation of benzene degrading organisms

In an effort to mimic the natural groundwater habitat, a site-specific medium consisting of filter

sterilized (0.2 micron, Corning) well water and noble agar (Difco^{TM}, BD) was prepareded. The final medium contained 75mL of 7.5% noble agar and 250mL of filter sterilized well 36 water and was mixed aseptically and poured into petri dishes. At the conclusion of the ^{12}C-benzene degradation, the well 36 treatments amended with 10 mM $(NH_4)_3PO_4$ treatments were sacrificed and used as the inoculum for the isolation experiment. Two dilution series (10^0-10^{-2}) were plated and spread in 100μL aliquots on the well water agar plates and incubated at 10°C in the presence and absence of benzene in a specially built incubation chamber. The incubation chamber consisted of a Rubbermaid® Servin' Saver 2.1 Gallon (7.8L) container with a Teflon coated-butyl septum fitted on top as a port for headspace gas sampling. The benzene source was 100 μL of liquid benzene placed in a crimp top-sealed-1.8 mL HPLC vial in the bottom center of the container. The HPLC vial was pierced with an 18-ga needle (PrecisionGlide^{TM}, BD) that remained lodged in septum to allow benzene vapor to freely disperse into the chamber. After 15 days of observation, small translucent colonies appeared on both the control and benzene exposed plates. At day 22, sterile colony transfer pads (RepliPlate^{TM}, FMC) were used to replica plate the cultures onto Stainer's Basal Salt Minimal Media agarose plates. Stainer's Basal Salts Minimal (BSM) media (broth and agarose) were prepared as per Burlage et al 1998. After 18 more days of incubation in benzene vapor the minimal media plates yielded sizable colonies. Nine colonies with distinct morphologies that grew only in the presence of benzene were selected and streaked for single isolation. Isolates were passaged 3 successive times in the presence of benzene and were picked and grown in R2A broth to be used as freezer stocks. Cultures were grown in R2A for 48hrs and 0.5 mL of culture was mixed with 0.5 ml 50% glycerol and stored at -80°C. Isolates were revived and streaked on BSM media and displayed growth only in the presence of benzene.

Phylogenetic analysis of benzene degrading isolates

The nine isolates were identified using 16S rRNA gene sequencing and ERIC-PCR genomic finger printing. 16S rRNA PCR was performed on individual colonies using 27F/1492R and ERIC primer sets (Table 1). The 25 µL 16s rRNA reactions included: 5µl 5x MyTaq^{TM} buffer (15 mM $MgCl_2$, 5mM dNTPs, (Bioline), 0.5 µL 27F/1492R primers (20µM), 0.1 µL MyTaq^{TM} DNA Polymerase, (5U/µL, Bioline) in thermocycler (MJ Research PTC-200) conditions: initial denaturation [95°C, 5min], 32 cycles of denaturation [94°C, 1 min], annealing [55°C, 1:30 min] and extension [72°C, 1:30 min], and a final extension [72°C, 10 min]. The 16S rRNA PCR amplicon was gel purified using QIAquick Gel Extraction Kit (Qiagen), ligated into a pCR2.1 plasmid vector (TOPO TA Cloning, Invitrogen) as per manufacturer's protocol. Plasmid were transform into chemically component *E. coli* (One Shot® TOP10, Invitrogen) cells and grown for blue/white screening. To verify the presence of the insert PCR with M13F/R primers (Invitrogen) was performed on white colonies and the amplicons were check with gel electrophoresis. Clones containing the insert were grown over night in Luria-broth with kanamycin (50 µg/µL) and the plasmids were extracted for sequencing using Zyppy^{TM} Plasmid Miniprep (Zymo). Sequencing was performed by Cornell University Life Sciences Core Laboratories Center, with an Applied Biosystems Automated 3730 DNA Analyzer using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. Consensus 16S rRNA sequences were built from 4 independently sequenced reactions using primer sets: M13F/M13R, 27F/1492R, 530F/519R, 1114F/1100R using ebiox (Version 1.5.1) (Table 1). The isolate sequences compared with GenBank nucleotide database library with BLASTn and Ribosomal Database Project for taxonomic identification. Phylogenetic trees were constructed using MEGA

5.05 (Kumar et al 2008). The isolate, clone and closest reference sequences were aligned with ClustalW and assembled using a neighbor- joining algorithm, with 1,000 bootstrap replications.

ERIC PCR genomic finger printing was conducted with ERIC2/ERIC1R (Versalovic et al 1991) primers (Table 1). PCR conditions included: 50 pmol each ERIC2 primer and ERIC1R primer (20µM), 0.4µL taq (5U/ µL), 4µL 15 mM $MgCl_2$, 1.25µL dNTPs (1.25mM), 5µL 5x Reaction Buffer, 2µL BSA (2ng/µL), 1 µL DNA template. Thermocycler conditions: Initial denaturation [95°C, 15 min] then 35 cycles of denaturation [94°C, 1 min], annealing [52°C, 1 min] and extension [65°C, 6 min], a single final extension [65°C, 16 min]. PCR products were run on 2% agarose gel (0.5x TBE) for 4hrs at 54v.

VarioRHD Primer Design

Primers were designed to target the aromatic-ring-hydroxylating dioxygenase beta subunit (Vapar_5383) of *Variovorax paradoxus* S110 S110 (NCBI reference sequence: NC_012792.1) using NCBI Primer-BLAST tool. The primers VarRHDF/VarRHDR amplify an 182bp region of the gene (Table 1). 25 µL PCR VarRHD reaction conditions included: 0.1µL Taq (5 U/µL) ™DNA Polymerase (Bioline), 5µL 5X MyTaq Buffer (15 mM $MgCl_2$, 5mM dNTPs, Bioline), 0.5µL 20µM VarioRHD F/R, 1 µL DNA Template. Thermocycler conditions: Initial denaturation [95°C, 10 min] then 40 cycles of denaturation [95°C, 1 min], annealing [60°C, 1 min] and extension [72°C, 30 sec], and a final extension [72°C, 10 min]. To validate the specificity of the primers the amplicon was gel purified with QIAquick® Gel Extraction Kit (Qiagen) cloned, sequenced and compared to the NCBI reference using Blastn.

Cell Extraction, RNA, RT-PCR and qPCR

Cells were sampled, concentrated by centrifugation (7000g) from the microcosm experiments and immediately frozen in liquid nitrogen. RNA Extractions were performed with Quick-RNA™

Table 2.1 Primers used in this study

Primer Name	Target Region	Sequence (5' - 3')	Source
M13F	N-terminal *LacZ* gene (Cloning Vector)	GTA AAA ACG ACG GCC AGT	Invitrogen
M13R	N-terminal *LacZ* gene (Cloning Vector)	CAG GAA ACA GCT ATG AC	Invitrogen
27F	Universal bacterial 16S rRNA gene	AGA GTT TGA TCC TGG CTC AG	Lane et al 1991
1492R	Universal bacterial 16S rRNA gene	GGT TAC CTT GTT ACG ACT T	Turner et al 1999
530F	Universal bacterial 16S rRNA gene	GTG CCA GCM GCC GCG G	Weisburg et al 1991
519R	Universal bacterial 16S rRNA gene	GTA TTA CCG CGG CTG CTG	Turner et al 1999
1114F	Universal bacterial 16S rRNA gene	GCA ACG AGC GCA ACC C	Turner et al 1999
1100R	Universal bacterial 16S rRNA gene	GGG TTG CGC TCG TTG	Turner et al 1999
ERIC2	Enterobacterial repetitive intergenic consensus regions	AAG TAA GTG ACT GGG GTG AGC G	Versalovic et al 1991
ERIC1R	Enterobacterial repetitive intergenic consensus regions	ATG TAA GCT CCT GGG GAT TCA C	Versalovic et al 1991
VarF	*Variovorax*-specific 16S rRNA gene	CAA TCG TGG GGG ATA ACG C	Bers et al 2011
VarR	*Variovorax*-specific 16S rRNA gene	GGC CGC TCC ATT CGC GCA	Bers et al 2011
VarRHD F	*V. Paradoxus* S110 aromatic-RHD beta subunit	GGA TGC CTT GCT GCG CTC GAT	This study
VarRHD R	*V. Paradoxus* S110 aromatic-RHD beta subunit	GCT GTT CGG TCT TGC GCC ACT	This study

(Zymo). The extraction buffer was applied directly to frozen pellet and the procedure was followed as per manufacturers protocol. Total nucleic acid extractions were treated with Dnase (Invitrogen) and converted to cDNA using SuperScript® III (Invitrogen) First Strand for Reverse Transcription. The cDNA was quantified using qPCR with dioxygenase-specific primers VarioRHDF/R and *Variovorax*-specifc 16S rRNA primers VarF/R (Bers et al 2011) (Table 1). Quantitative PCR was performed on an Applied Biosystems 7300 Real Time PCR System. Standards were made by serial dilution of gel purified VarF/R and VarioRHDF/R PCR amplicons and comparing them with known quantities of lambda DNA using Quant-iT™ (PicoGreen® dsDNA reagent, Invitrogen P7581). The 16S rRNA and RHD reactions were run under PCR conditions including 12.5 µL Master Mix (SYBR Select, Applied Biosystems), 0.3 µL RHD/16S (20uM) 240nM, 0.3 µL VarR (20uM) 240nM, 10.9 H_2O, 1.0 µL template cDNA. Quantitative PCR thermocycler conditions included 2min at 50°C, 15min at 95°C, 40 Cycles (15 sec at 95°C, 30sec at 58°C), and a disassociation curve was included to assess amplification specificity.

***Metabolite Analysis of Variovorax* MAK3**

500 mL *Variovorax* MAK3 and *Pseudomanas Putida* F1 cells were grown on benzene. A 5 mL concentrated cell suspension was extracted with neutralized ethyl acetate, derivatized with butylboronic acid (BBA) and analyzed by gas chromatography/mass spectrometry for cis-dihydrodiol and catechol as per Pumphrey and Madsen 2007.

Gas Chromatography/Mass Spectrometry Analysis of Benzene

A Hewlett-Packard HP6890 gas chromatograph (Wilmington, DE) linked with a HP 5973 mass selective detector was used to quantify benzene. The gas chromatograph (GC) was fitted with a Hewlett-Packard HP-5 phenylmethyl-siloxane capillary column (Model no. HP 19091J-

433, Capillary 30.0m x 250 um x 0.25 um), carrying high-purity helium gas supplied by Airgas (Elmira, NY). The GC parameters included, a spilt injection (20:1), inlet temperature at 150°C, initial GC oven temp was at 50°C and ramped 15°C /min to a final temperature of 100°C. The mass spectrometer detector was operating at 2000eV and a vacuum of 2 x 10^{-5} tor, in scan mode from m/z 50 to 500 (benzene m/z = 78). Calibration curves were constructed using external standards with known amounts of benzene (EMD, Germany) vapor. Benzene concentrations were averaged and compared by standard deviation from triplicate chambers. Benzene was measured by taking 100 µL headspace samples with a 250µL gas tight syringe (Hamilton, NV).

2.3 Preliminary/Foundational data

This investigation examined the ability of microbial communities in coal tar-contaminated groundwater to degrade benzene and identified active populations. Microcosm incubations were designed to assess the potential for benzene degradation with and without added nutrients (N and P). Samples were taken from monitoring wells in the contaminated aquifer study site and used in laboratory incubations. Parallel experiments were conducted using samples from two monitoring wells (12 and 36) with distinct oxygen saturation profiles (Yagi et al 2010). Historical records have established that the water and sediment matrix-surrounding well 12 was less contaminated and more oxic than that in well 36, which is more centrally located in the contaminant plume and had higher levels of concentrations of aromatic hydrocarbons (Bakermans et al 2002, Neuhauser et al 2009, Yagi et al 2010). Detailed records of site geochemistry can be found in Neuhauser et al (2009) and Yagi et al (2010).

Microcosm incubations were conducted to assess the ability of the microbial community in the waters to degrade benzene. Water samples from the two wells were dosed with 3 ppm

benzene and apportioned into three treatments: (i) site water only (unamended) (ii) amended with10% v/v sterile-sediment and (iii) amended with 10 mM $(NH_4)_3PO_4$. Gas chromatography/mass spectrometric analysis (GC/MS) of headspace gases in the microcosms was used to quantify the concentration of benzene over time. As expected, benzene persisted in the abiotic treatments for wells 36 and 12 (Figures 2.1 and 2.2, respectively) during the >300-h incubation. Surprisingly, benzene also persisted in the unamended well water for >350 h. The lack of benzene biodegradation in the unamended incubation was demonstrated to be a result of nutrient limitation, since site-associated sources were capable of supporting benzene metabolism by the native community. The community in the sediment- and nutrient-amended treatments from well 36 degraded the benzene completely in 300 and 360 hours, respectively (Figure 2.1). Similarly, the community from well 12 degraded benzene in 200 and 250 hours for nutrient and sediment amended microcosms, respectively (Figure 2.2). Clearly N and P sources associated with the site sterile aquifer solids are capable of supporting benzene metabolism by the native community, These results demonstrate that the microbial communities in site waters are capable of degrading benzene, but that N and/or P limitation can occur if site waters are incubated in the absence of site aquifer solids. Thus, all sediment-free degradation assays should include the N/P supplement. We next sought to identify the microbial community members responsible for benzene degradation.

Stable isotope probing in coal tar contaminated well water microcosms

To identify the members of the aquifer microbial community that are active in benzene metabolism we performed a stable isotope probing (SIP) experiment in parallel with the degradation assays (Figures 2.1 and 2.2). Treatments were prepared identically as the degradation assay except ^{13}C-labelled liquid benzene was added as the carbon source in

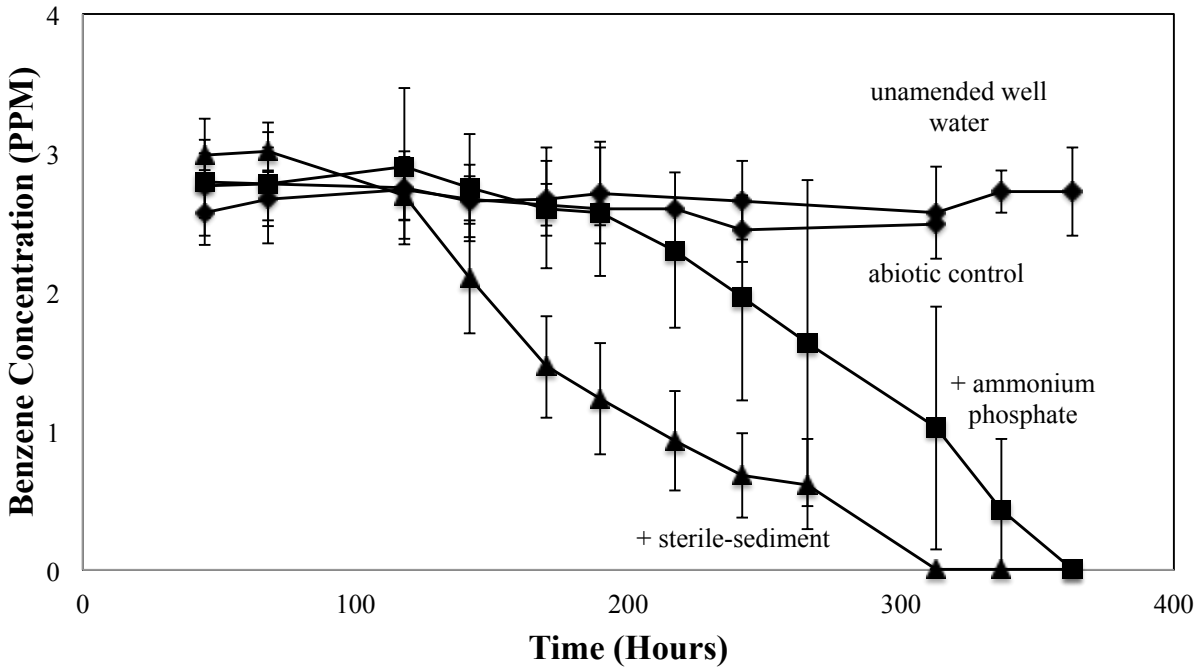

Figure 2.1. Benzene biodegradation in serum-bottle incubations containing well 36 microorganisms. Gas chromatography/Mass spectrometric analysis of headspace gases was used to determine the concentration of benzene at each time point. The experiment included 4 treatments: Sterile-sediment-amended (10% v/v), 10 mM $(NH_4)_3PO_4$-amended, no amendment, and an abiotic control (HCl to pH2). Each data point represents triplicate treatments and error bars represent standard deviations. The final two times for the abiotic control were not sampled.

the microcosms. After 70% of the headspace ^{13}C-labelled benzene was degraded the microcosms were sacrificed and the ^{13}C-labelled DNA fraction was isolated (Jeon et al 2003) and amplified using universal bacterial 16S rRNA primers (Lane et al 1991, DeRito et al 2005). The 16S rRNA PCR was ligated into pCR2.1 plasmid vector (TOPO TA Cloning, Invitrogen) and transformed into chemically component *E. coli* (One Shot® TOP10, Invitrogen). White colonies were picked and analyzed using RFLP was used to determine 14 unique profiles which were sent for sequencing, 10 from Well 36 and 4 from Well 12. A phylogenetic analysis of the 14 SIP-generated clones representing the active benzene population is shown in figure 2.3. The resulting SIP sequences were aligned with nearest genbank reference determined by blastn in MEGA 5 using ClustalW. The phylogenic analysis showed that γ- and β-Proteobacteria, specifically the genera *Acidovorax*, *Variovorax* and *Pseudomonas,* as active-benzene degrading microogranisms. Results from the clone library reveled that well 36 was highly enriched with 2 sequences of *Variovorax* which together comprised 42% of the clone library and one *Acidovorax* sequence represented another 49% of the library (Figure 2.3).

Results of stable isotope probing (SIP) procedures provided major insights into the identities of the active benzene degrading populations, but SIP assays are known to have organisms not responsible for the primary degradation of the compounds of interest, and (ii) failure to adequately separate the DNA of metabolically active (^{13}C-labled) populations from the background (inactive, unlabeled) community members (DeRito et al 2005, Dumont and Murrell 2005, Madsen 2006).

We conducted additional investigations to test if *Variovorax* and *Acidovorax* spp implicated by the SIP experiment were responsible for benzene degradation in site water microcosms by linking expression of the particular dioxygenase gene to the *Variovorax* spp

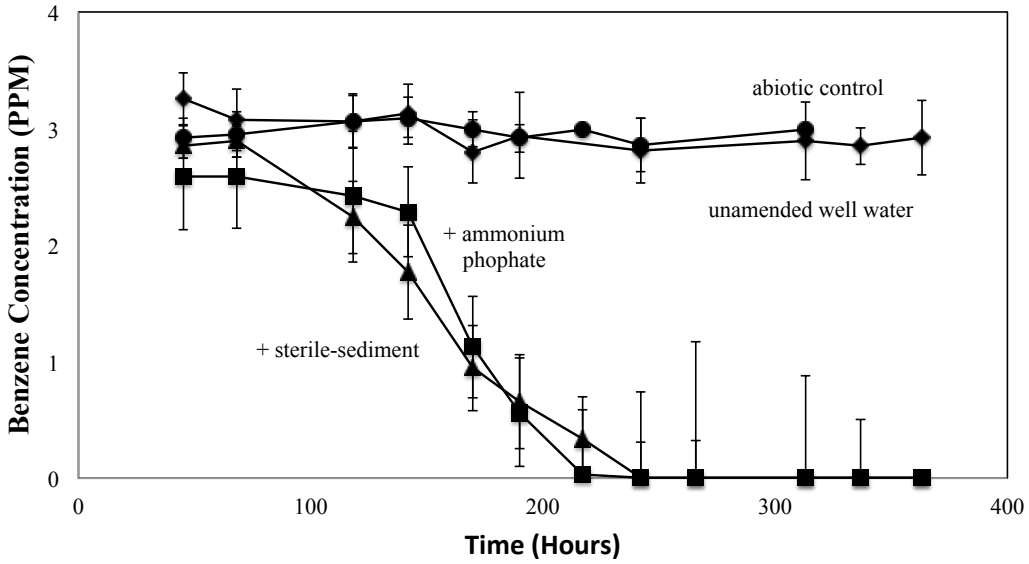

Figure 2.2. Benzene degradation in serum-bottle incubations containing well 12 microorganisms. Gas Chromatography/Mass Spectrometry analysis of headspace gases determined the concentration of benzene at each time point. The experiment included 4 treatments: Sterile well sediment-amended (10% v/v), 10 mM $(NH_4)_3PO_4$-amended, no amendment and an abiotic control (HCl to pH 2). Each data point represents triplicate treatments and error bars represent standard deviations. Final two times for killed control were not sampled.

Figure 2.3. Identities of active benzene-degrading well water bacteria found by two stable isotope probing experiment. ^{13}C-Benzene was metabolized by microorganisms in the 10 mM $(NH_4)_3PO_4$-amended treatments from well 36, and well 12 (see figures 2.1 and 2.2). After DNA extraction and CsCl-separation of ^{13}C-DNA from ^{12}C-DNA, the former was used for preparation of clone libraries of 16S rRNA genes. A maximum likelihood tree of full (>1400bp) bacterial 16S rRNA gene sequences was prepared from ^{13}C-labeled DNA. The sequences were aligned using ClustalW and the tree was constructed using MEGA 5.05. Bootstrap values estimate node confidence. Ten distinctive clones from well 36 (bold, with "36" prefix) and four from well 12 (bold, with "12" prefix) were derived from the experiment. Ten reference sequences are included in the tree; the out-group is *Methanosarcina barkeri*.

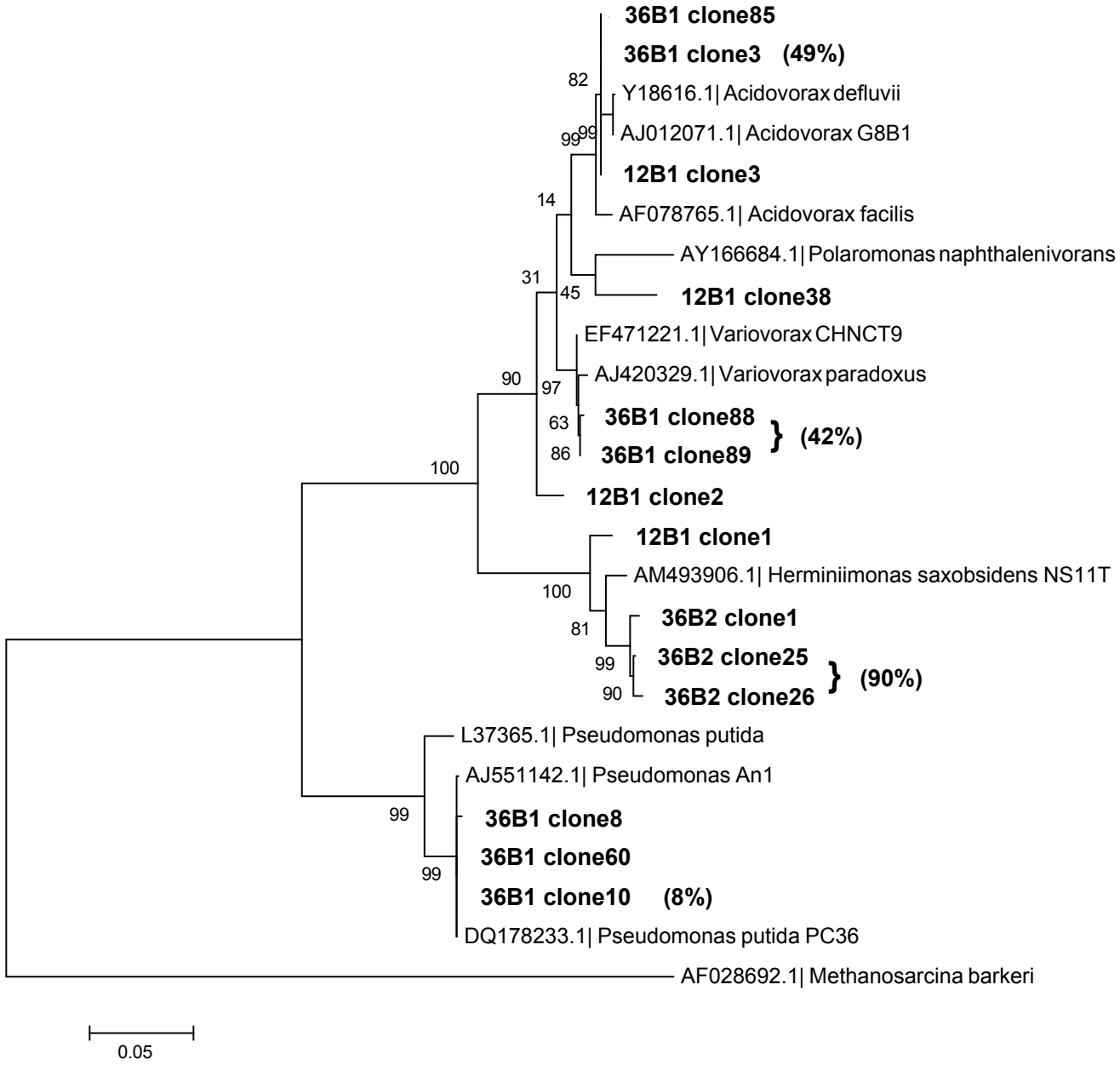
36B1 clone85
36B1 clone3 (49%)
82
Y18616.1| Acidovorax defluvii
99
99
AJ012071.1| Acidovorax G8B1
12B1 clone3
14
AF078765.1| Acidovorax facilis
AY166684.1| Polaromonas naphthalenivorans
31
45
12B1 clone38
EF471221.1| Variovorax CHNCT9
90
97
AJ420329.1| Variovorax paradoxus
63
36B1 clone88
86
36B1 clone89
(42%)
100
12B1 clone2
12B1 clone1
AM493906.1| Herminiimonas saxobsidens NS11T
100
81
36B2 clone1
99
36B2 clone25
90
36B2 clone26
(90%)
L37365.1| Pseudomonas putida
AJ551142.1| Pseudomonas An1
99
36B1 clone8
36B1 clone60
99
36B1 clone10 (8%)
DQ178233.1| Pseudomonas putida PC36
AF028692.1| Methanosarcina barkeri
0.05

Figure 2.3

observed in the microcosms to whole-community benzene biodegradation.

2.4 Results

Isolation of benzene degrading organisms using benzene vapor

To validate the results of the SIP experiment, we sought to obtain cultivated representatives of the benzene-degrading organisms. At the conclusion of the benzene degradation assay shown in Figure 2.1, the well 36 $(NH_4)_3PO_4$-amended incubations were used as inoculum for cultivation and isolation procedures. We built a benzene diffusion chamber that provided benzene vapor as the sole carbon and energy source to well-water bacteria plated onto the agar media. To mimic nutritional condition of the field site, the agar medium was prepared with 77% filter-sterilized well-36 water. A dilution series (10^0-10^{-2}) of the incubation water was plated in 100µL aliquots on to this medium for the first phase of obtaining benzene-degrading isolates.

The plates were incubated in the presence and absence of benzene. The benzene headspace concentration averaged from 10 to 15 ppm during the incubations. After 15 d of incubation, small translucent colonies appeared on both the control and benzene exposed plates. After an additional week of incubation without noticeable growth, nitrocellulose pads were used to replicate the colonies from benzene-exposed plates onto Stainer's Basal Salts Minimal (BSM) media (Burlage et al 1998). The benzene-exposed replicates yielded sizable colonies within 18 d, while over the same time period no growth was observed in control incubations lacking the benzene vapor. Nine colonies exhibiting distinct morphologies were repeatedly streaked onto the BSM + benzene vapor plates until pure cultures were obtained. The final cultures yielded robust colonies in the presence but not in the absence of benzene (Figure 2.4). To ensure each culture

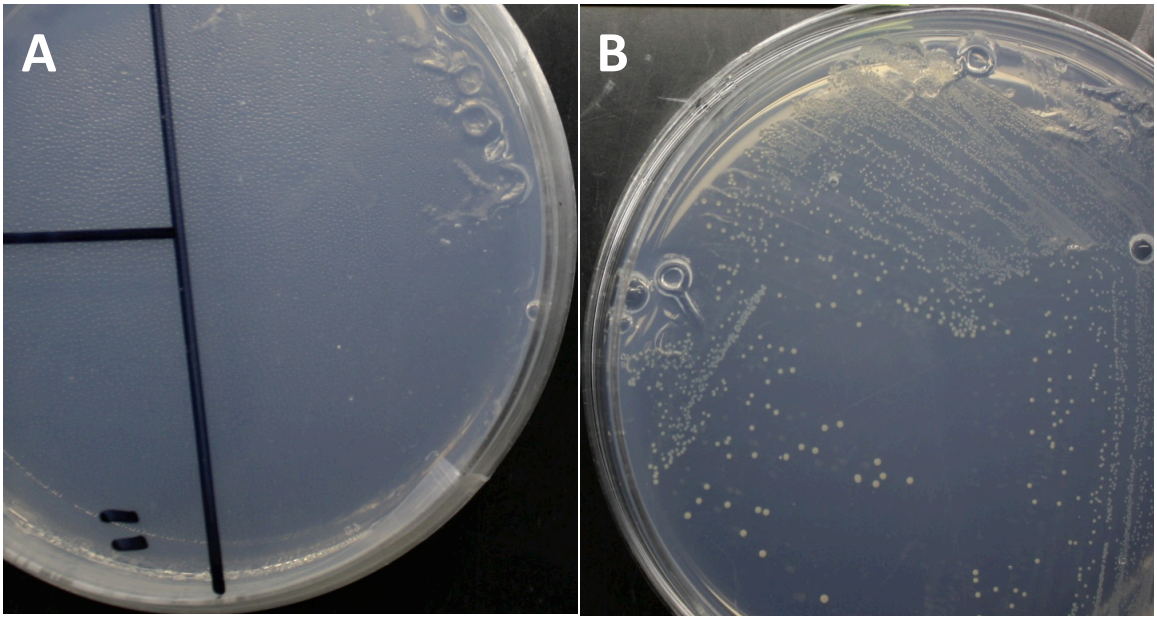

Figure 2.4 Images of isolate MAK3 grown on minimal media plates in the absence (A) and presence (B) of benzene vapor. The results demonstrate growth of isolate MAK3 after seven days at 21°C in an average headspace benzene concentration of approximately 15 ppm (B), and no growth in the absence of benzene (A). Headspace benzene concentration was monitored by Gas Chromatography/Mass Spectrometry (see Appendix 1).

was genetically distinct (i.e. avoiding duplicates) repetitive extragenic palindromic–enterobacterial repetitive intergenic consensus (REP-ERIC) PCR (Versalovic et al 1991) was performed to further distinguish the isolates from one another; each isolate displayed a unique banding pattern (Figure 2.5). We sought to confirm that individual isolates would exhibit the same benzene utilizing phenotype in liquid media that was observed by colony formation on agar media. To test this the nine isolates were grown in BSM broth at room temperature with benzene as the sole carbon source. Five of the nine isolates successfully degraded benzene over the incubation period of 70 hours (Figure 2.6). The remaining isolates including the control did not show significant loss of benzene over the same time. We speculate that the four isolates may not have degraded benzene in liquid culture because of the toxicity of dissolved benzene at 3 ppm or carbon scavenging from the agar.

16S rRNA gene Sequencing of Isolates revealed high similarity to benzene degrading bacteria identified in cultivation-independent SIP studies

Universal bacterial 16S primers 27f, 1492r (Weisenburg et al 1991) were used to amplify the 16S rRNA gene from each of the nine isolated benzene degrading bacteria. The amplicons were gel purified, cloned, sequenced, and assembled to create full (>1400bp) 16S rRNA gene sequences. The isolate sequences were compared with those derived from the ^{13}C-labelled DNA fraction of the stable isotope probing experiment (Figure 2.7). The results indicated that several of t isolates shared >97% similarity to SIP clones. Most notably isolate MAK5 had 97% identity with a SIP clone (36B1 clone3), which comprised 49% of clone library and MAK3, shared 98% with two SIP sequences (36B1 clone88 and 36B1 clone89) that comprised 42% of the clone library. The largest group of isolates sequences clustered with genus *Rhodococcus*, which was not identified in the SIP experiment.

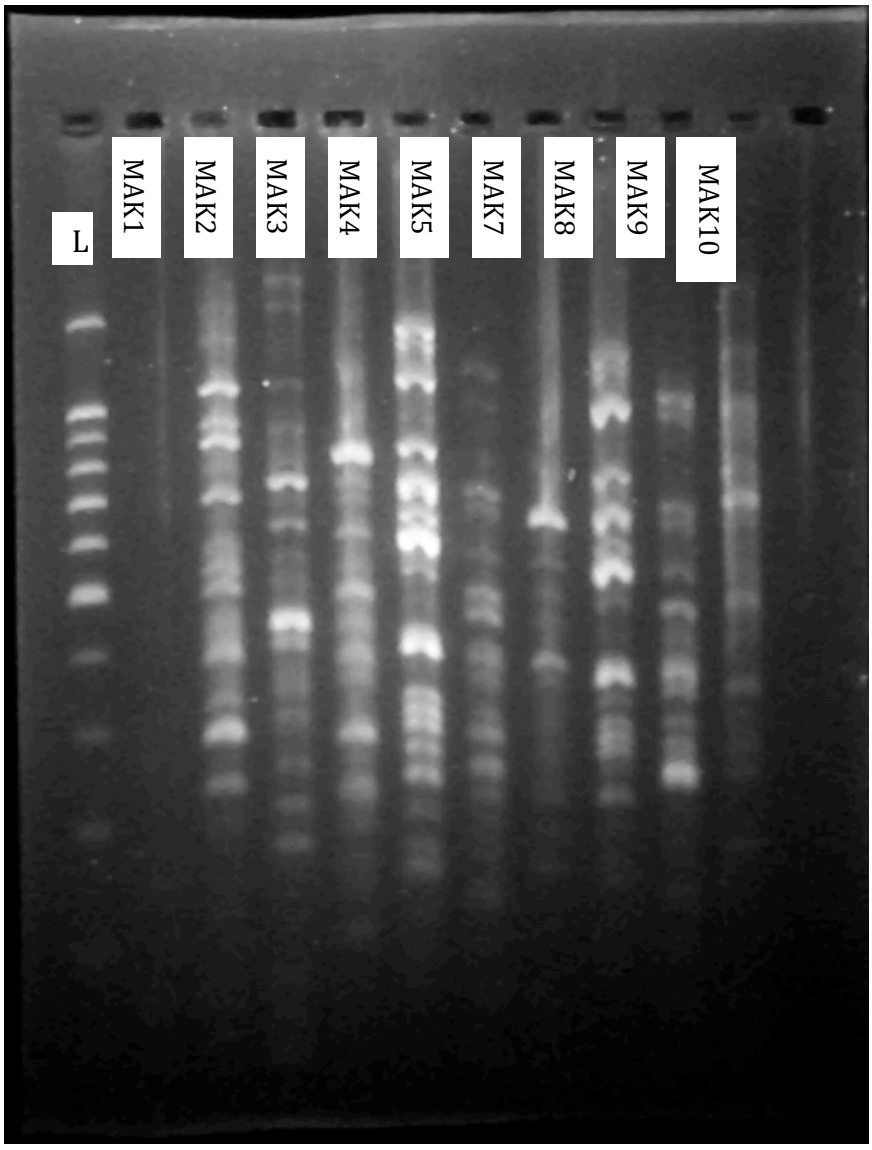

Figure 2.5. Genetic fingerprinting (ERIC-PCR) of the nine benzene-degrading confirmed their distinctness.

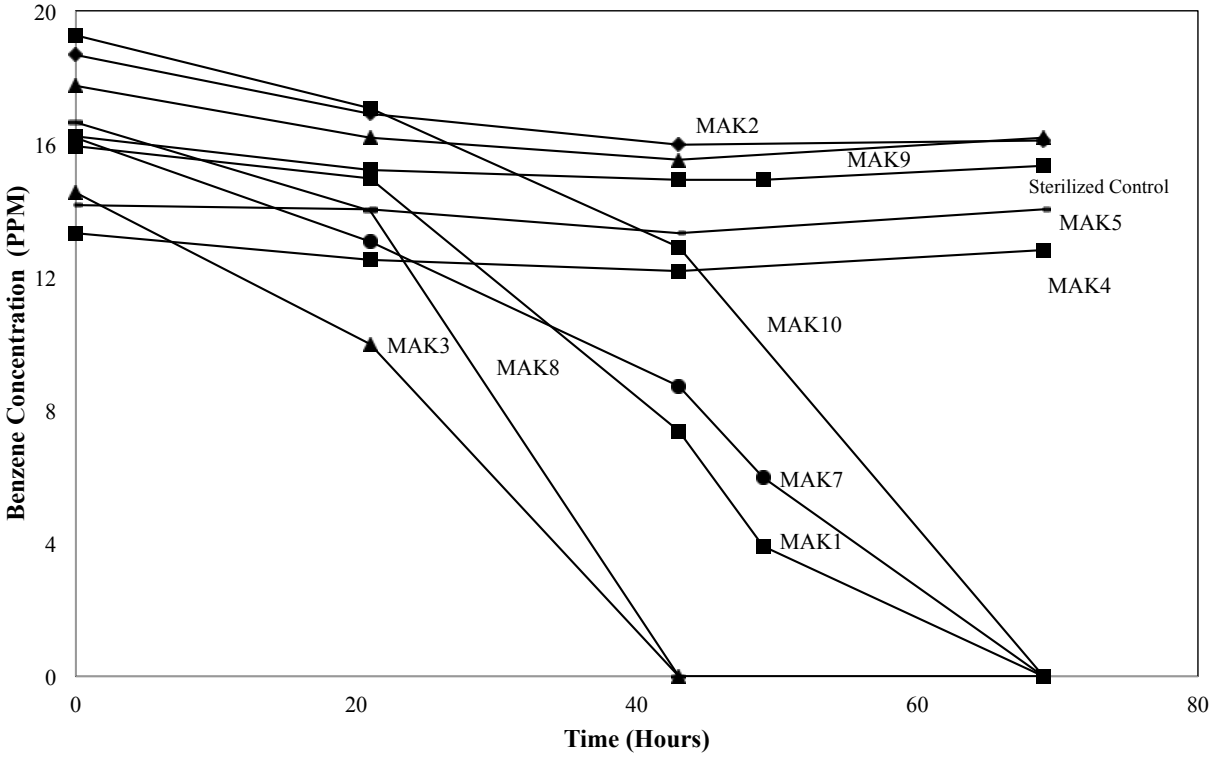

Figure 2.6. Physiological tests for benzene degradation by nine pure cultures of isolated bacteria in minimal media. Single colonies were placed in BSM broth and dosed with 3PPM benzene, incubation at 21° C and shaken at 120 rpm. Gas Chromatography/Mass Spectrometry analysis of headspace gases determined the concentration of benzene at each time point. Each time point represents a single measurement.

The similarity of 16S rRNA phylogeny of isolates MAK3 and MAK5 to the *Acidovorax* and *Variovorax* (respectively) to 16S rRNA sequences observed in the SIP study suggested that the cultivation strategies successfully isolated representatives of the community that were previously identified by SIP to be active benzene degradation in site waters.

Additional verification of benzene degradation by isolates MAK3 and MAK5 in minimal liquid media

An additional experiment was performed on the isolates most similar to the clones found from the SIP experiment, MAK3 and MAK5. The two isolates were inoculated into BSM broth. Initial cell densities were 6 x 10^6 CFU/Ml with liquid benzene added at a concentration of approximately 12 ppm. *Variovorax* MAK3 consumed >90% of benzene in 96 hours and *Acidovorax* MAK5 required approximately 160 hours (Figure 2.8). During benzene degradation cells were concentrated and extracted for metabolite analysis. In the control culture containing *Pusedomons putida* F1, we were able to detect the presence of cis-1,2-dihydrodiol and catechol. In the culture containing *Variovorax* MAK3 we did not detected cis-1,2-dihydrodiol, but were able to detect phenol and catechol. We next sought to identify the genes responsible for degradation.

***Variovorax* dioxygenase gene expression during benzene degradation**

Isolate MAK3 was chosen for further investigation because high representation in the SIP clone library (Figure 2.3). We designed qPCR primers based on a putative dioxygenase gene annotated in the *Variovorax paradoxus* S110 genome. We verified the specificity of the primers using conventional PCR on isolate MAK3 and sequenced the 182 bp amplicon. The results confirmed 96% identity to the annotated dioxygenase gene in *Variovorax paradoxus* S110 (Figure 2.9).

We hypothesized that the *Variovorax* MAK3's ring hydroxylating dioxygenase (RHD) gene would be upregulated (highly expressed) by the culture. To test this we prepared microcosm incubations in the presence and absence of benzene and two negative-controls: one with no added carbon and the other with glucose as the growth substrate. Over the course of the experiment (Figure 2.10), biomass was subsampled at times corresponding with measurements of benzene concentration. RNA extraction allowed quantification of dioxygenase transcript abundance. The period of most-active benzene metabolism by *Variovorax* MAK3 cells was between 21 and 27 hours (14%. to 57% benzene depletion). During this same period, the qRT-PCR results of the showed that RHD transcript abundance increased ten-fold between 21hr and 27hr (Figure 2.10). The elevated transcript levels persisted to the final time point when 98% of the benzene had been consumed. Absolute transcript copies of the MAK3 RHD gene increased from approximately 1.0×10^3 copies/ng at 14% benzene depletion to 1.3×10^4 copies/ng at 57% benzene depletion and 1.0×10^4 copies/ng at 98% depletion. Over the same time periods the RHD transcript levels in the glucose did not increase and the levels were similar to those in the no-benzene control.

Additionally, Variovorax-specific 16S rRNA primers (Bers et al 2011) were used to monitor by qPCR, the relative abundance of the inoculated cells during the experiment. The results indicate that 16S rRNA copies increased 150% in the benzene treatment, while the treatment without benzene decreased by 62% during the experiment. The glucose treatment demonstrated the largest increase, 16S rRNA copies rose 410%.

These results show that an increase in the dioxygenase transcript abundance corresponded to the rapid degradation of benzene and an increase in 16s rRNA copy number, associated with cell growth. The comparison of the dioxygenase results between the benzene and

Figure 2.7. Phylogenetic affiliations of both cultivated benzene-degrading bacteria and 16S rRNA clones derived from the Stable Isotope Probing (SIP) study shown in Figure 2.3. Maximum-likelihood tree of full (>1400bp) bacterial 16S rRNA genes from the culture-derived isolates and clones from 10 mM $(NH_4)_3PO_4$-amended ^{13}C-benzene SIP experiment from both monitoring wells 12 and 36 using MEGA 5.05. Bootstrap values estimate node confidence. The comparison consisted of the nine isolate sequences (underlined), ten clones (in bold) from well 36 and four from well 12. Nineteen reference sequences are included in the tree; the outgroup is *Methanosrcina barkeni*.

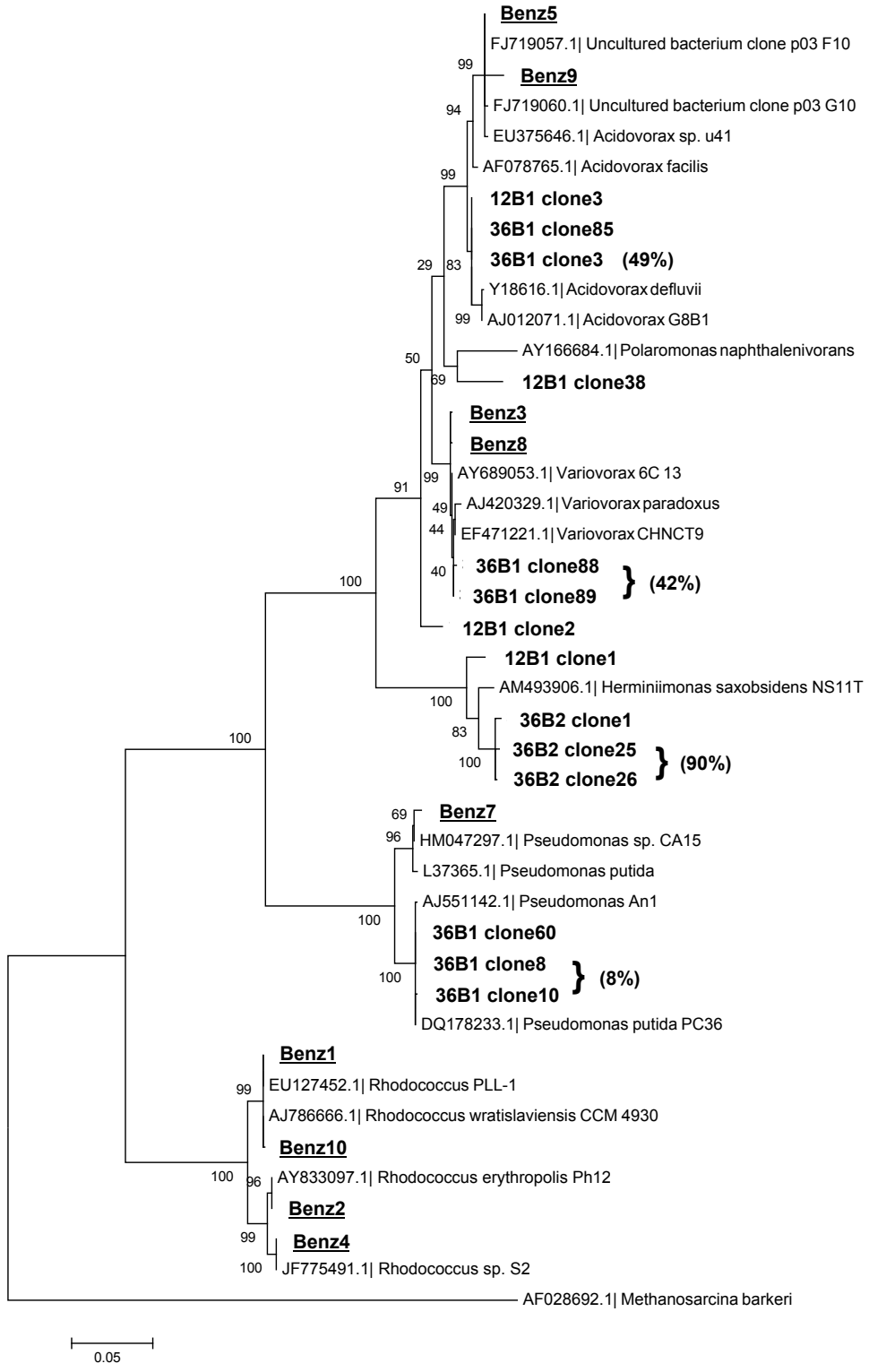
Benz5
FJ719057.1| Uncultured bacterium clone p03 F10
Benz9
FJ719060.1| Uncultured bacterium clone p03 G10
EU375646.1| Acidovorax sp. u41
AF078765.1| Acidovorax facilis
12B1 clone3
36B1 clone85
36B1 clone3 (49%)
Y18616.1| Acidovorax defluvii
AJ012071.1| Acidovorax G8B1
AY166684.1| Polaromonas naphthalenivorans
12B1 clone38
Benz3
Benz8
AY689053.1| Variovorax 6C 13
AJ420329.1| Variovorax paradoxus
EF471221.1| Variovorax CHNCT9
36B1 clone88
36B1 clone89
(42%)
12B1 clone2
12B1 clone1
AM493906.1| Herminiimonas saxobsidens NS11T
36B2 clone1
36B2 clone25
36B2 clone26
(90%)
Benz7
HM047297.1| Pseudomonas sp. CA15
L37365.1| Pseudomonas putida
AJ551142.1| Pseudomonas An1
36B1 clone60
36B1 clone8
36B1 clone10
(8%)
DQ178233.1| Pseudomonas putida PC36
Benz1
EU127452.1| Rhodococcus PLL-1
AJ786666.1| Rhodococcus wratislaviensis CCM 4930
Benz10
AY833097.1| Rhodococcus erythropolis Ph12
Benz2
Benz4
JF775491.1| Rhodococcus sp. S2
AF028692.1| Methanosarcina barkeri
0.05

Figure 2.7

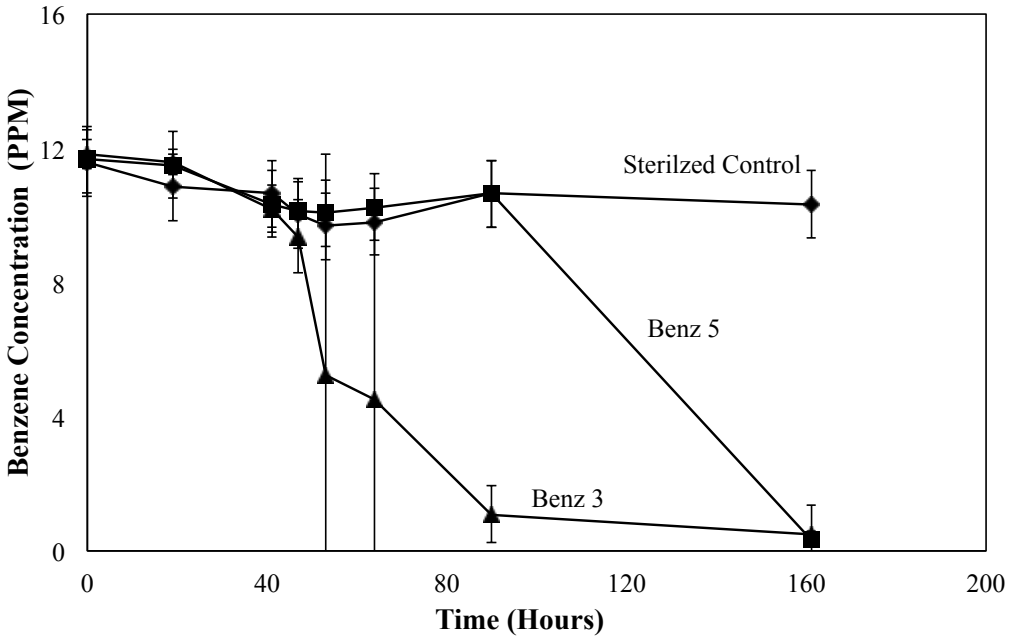

Figure 2.8. Confirmation of benzene degradation by isolates MAK3 and MAK5 in serum-bottle laboratory incubations. GC/MS analysis of headspace gases determined the concentration of benzene at each time point. Isolates MAK3 or MAK5 were inoculated (initial cell density ~10^6 cfu/mL) into minimal medium with liquid benzene added at an initial concentration of 12ppm. Each data point represents triplicate treatments and error bars represent standard deviation.

glucose treatments strongly suggest that the putative dioxygenase gene is specifically involved in the catabolism of benzene and not in general growth or metabolism. Furthermore, the difference in 16S rRNA values found in the presence versus the absence of benzene confirm that *Variovorax* MAK3 can derive its carbon and energy solely from benzene.

The final series of experiments were designed to test the hypothesis that the Variovorax-related, naturally-occurring populations are active in the degradation of benzene in the microbial community native the site's groundwater. The 16S rRNA and RHD gene qPCR assays described above were applied to N-and-P amended well 36 incubations with and without added *Variovorax* MAK3 cells, with and without added benzene. Samples were sacrificed for biomass extraction at times in the experiment when approximately 0%, 25% and 90% of the initial benzene concentration was consumed.

The addition of *Variovorax* MAK3 accelerated benzene substantially (degradation time 3-fold) compared to the native community alone (Figure 2.11A). The isolate-amended treatment consumed ~100% of the benzene in 90 hours, while the native community required 300h to degrade the benzene present. In the inoculated treatment, absolute transcript abundance for the MAK3 RHD gene increased over time corresponding well with the accelerated loss of benzene during the first 100 hours of the experiment (Fig. 2.11B); RHD transcripts were not detected initially but increased to 246 copies/ng when 25% of benzene had been consumed and 362 copies/ng when 90% had been consumed. These patterns in RDH gene expression are consistent with pure culture experiment (Figure 2.10) and indicate that benzene loss is mechanistically tied to RDH gene activity. Clearly, *Variovorax* MAK3 is adapted to degrading benzene in site waters.

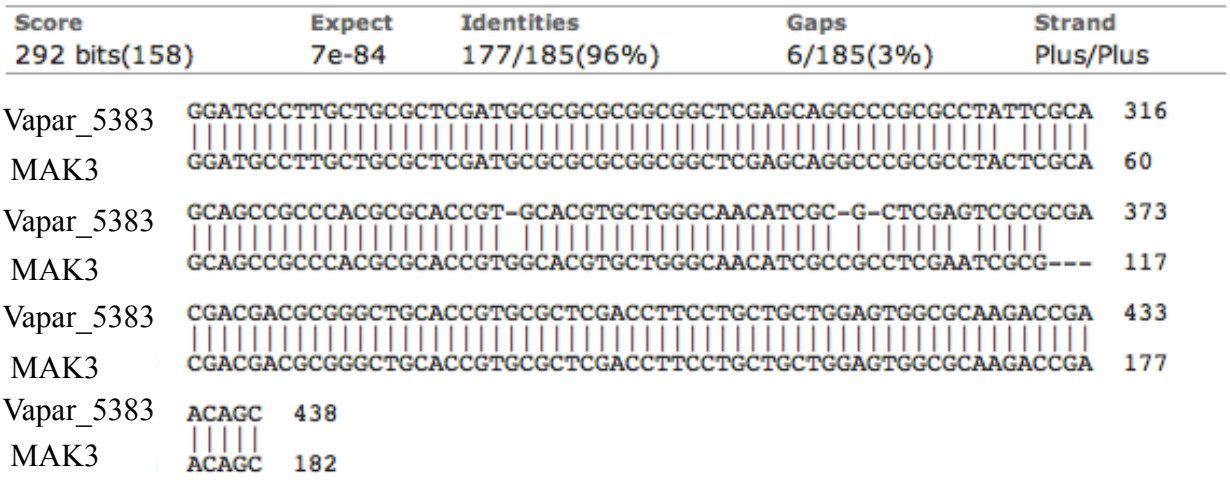

Figure 2.9. Alignment of Locus_Tag Vapar_5383 the 182 bp portion of the aromatic-ring-hydroxylating dioxygenase beta subunit sequence from the complete genome of *Variovorax Paradoxus* S110 (NCBI reference sequence: NC_012792.1) and the corresponding (MAK3) sequence found in isolate *Variovorax* MAK3. Blastn comparison showed 96% similarity between the two sequences.

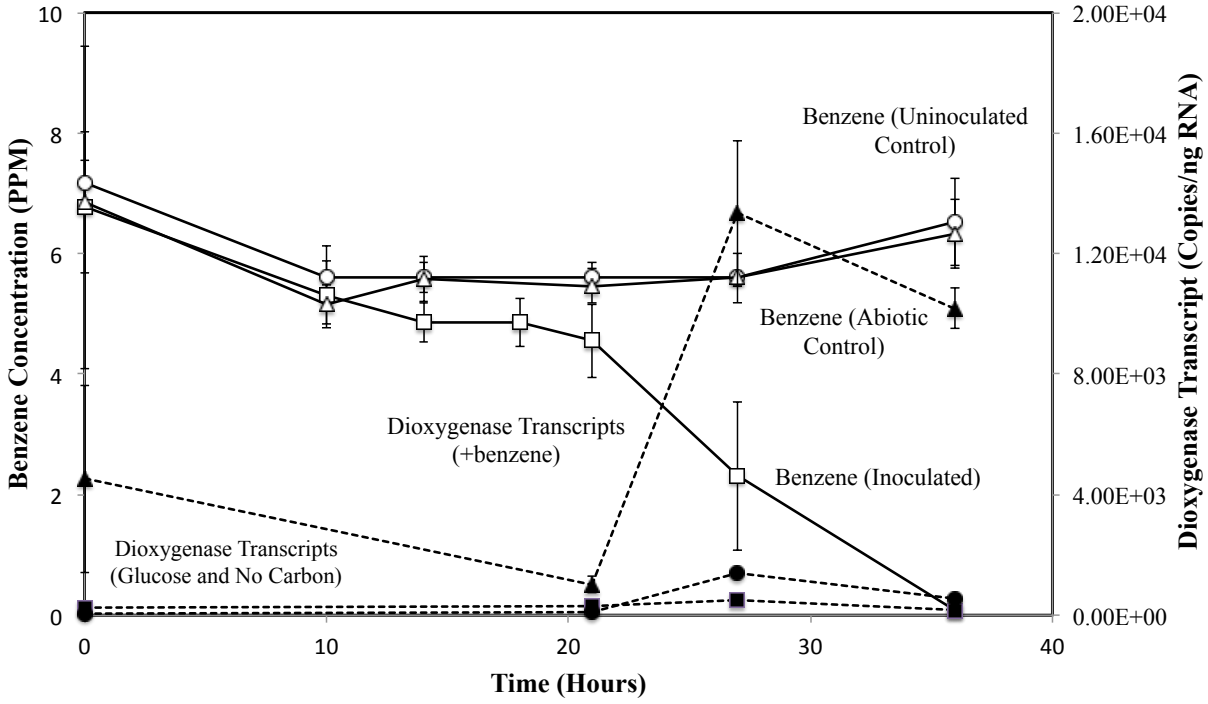

Figure 2.10. Benzene degradation by isolate *Variovorax* MAK3 and corresponding mRNA transcript abundance of this organism's beta subunit ring hydroxylating dioxygenase (RDH). Incubations in minimal media consisted of four treatments (initial cell density ~10^6 cfu/mL): (i) cells in the presence benzene (ii) cells in the presence of glucose (iii) abiotic control (HCl) (iv) cells in the absence benzene. Benzene degradation, quantified by GC/MS, is shown in the open symbols. Dioxygenase transcript abundance, quantified by RT-PCR, is represented by the closed symbols. Each data point represents triplicate treatments and error bars represent standard deviation.

Variovorax MAK3 and its expressed dioxygenase gene accelerate benzene degradation in the inoculated and uninoculated groundwater microbial community

Finally (Figure 2.11C), we show in the uninoculated treatment that benzene induces expression of Variovorax-related RHD mRNA transcripts over the course of benzene degradation by the groundwater community. Populations of *Variovorax* native to the groundwater were quantified by qPCR of 16s rRNA genes; so also were mRNA transcripts of the *Variovorax*-associated RDH gene. When the dioxygenase gene transcripts were normalized as a ratio of RDH expression to Variovorax-related detected 16S rRNA, the treatment that received benzene demonstrated a clear increase in RDH gene expression (Figure 2.11C). Expression increased over 6 fold as the ratio of RDH dioxygenase to 16S rRNA copies; from a ratio of 13 to 87 over the course of the assay (Figure 2.11A) during which the native, uninoculated groundwater community successfully carried out benzene biodegradation.

2.5 Discussion

The interpretation of these results must be tempered with an understanding of the limitations of the methodologies utilized in this investigation. Our aim was to use groundwater microcosms as a model system to investigate benzene degradation by microbial communities in coal-tar contaminated groundwater. Although benzene is a minor constituent of coal-tar, the continued presence of other BTEX compounds on site and the ubiquity of benzene as an environmental pollutant make it a relevant model compound (Neuhauser et al 2009). Despite our attempts to mimic naturally occurring field conditions in our incubations we acknowledge that the behaviors and composition of microbial communities in laboratory incubations are not necessarily indicative of their natural state or functions in the environment (Madsen 1998).

Figure 2.11. Experiments designed to assess the role of *Variovorax* strain MAK3 and its ring-hydroxylating dioxygenase (RHD) gene in benzene degradation in the naturally-occurring well water microbial community. Panel A. Benzene degradation in freshly gathered contaminated well water sample (Well 36) with and without added *Variovorax* strain MAK3 cells (initial cell density ~10^5 cfu/mL). Panel B. Dioxygenase transcript abundances (β-subunit of ring hydroxylating dioxygenase; see Figure 2.9) in well 36 well water community with and without added *Variovorax* strain MAK3 (cells initial cell density ~10^5 cfu/mL). C. Induction of dioxygenase mRNA (β-subunit of ring hydroxylating dioxygenase) in the well 36 microbial

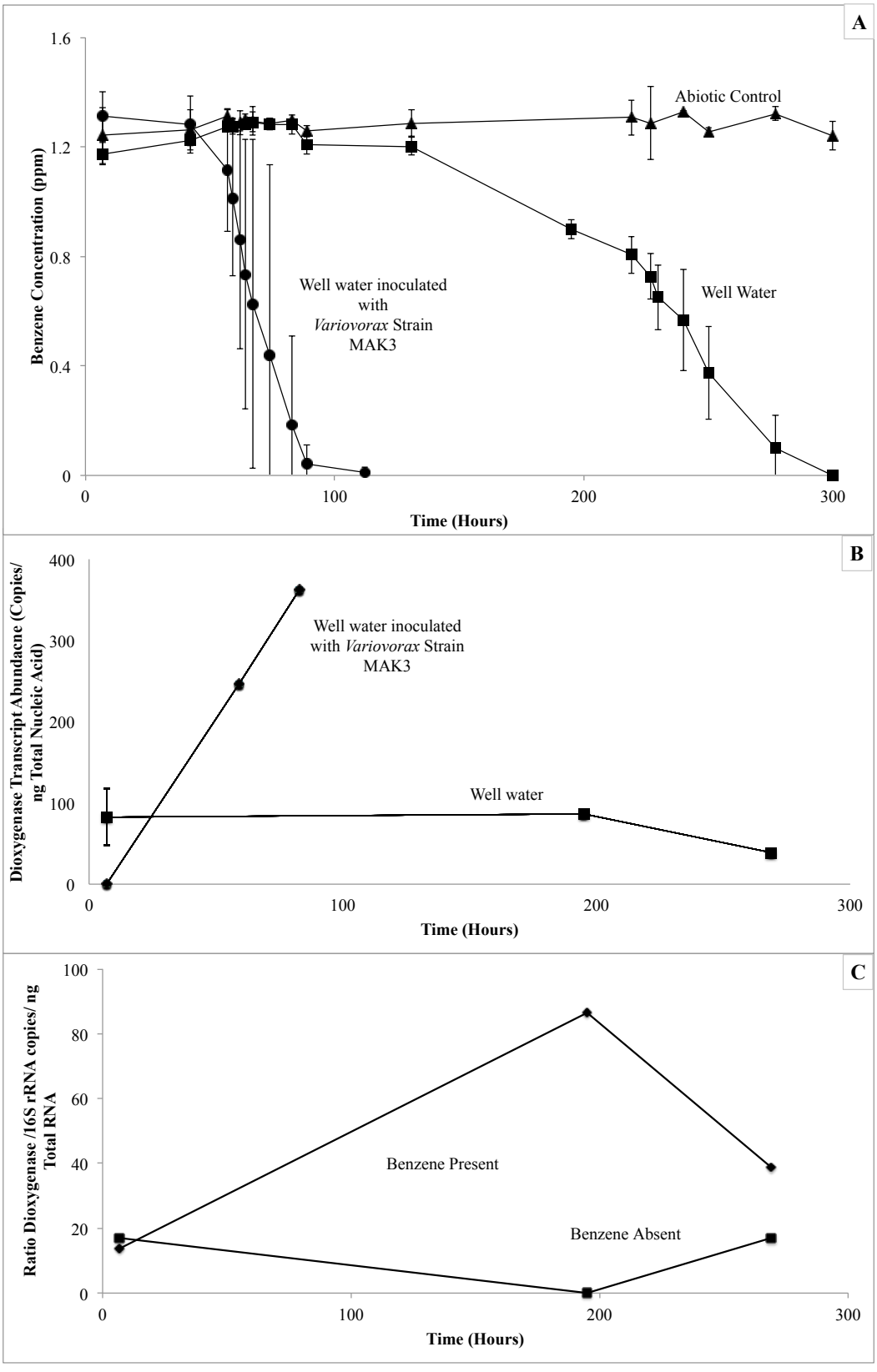
A
Benzene Concentration (ppm)
1.6
1.2
0.8
0.4
0
0
100
200
300
Time (Hours)
Abiotic Control
Well water inoculated with Variovorax Strain MAK3
Well Water
B
Dioxygenase Transcript Abundacne (Copies/ ng Total Nucleic Acid)
400
300
200
100
0
0
100
200
300
Time (Hours)
Well water inoculated with Variovorax Strain MAK3
Well water
C
Ratio Dioxygenase /16S rRNA copies/ ng Total RNA
100
80
60
40
20
0
0
100
200
300
Time (Hours)
Benzene Present
Benzene Absent

Fig. 2.11

community resulting from benzene addition. *Variovorax* strain MAK3 cells were not added to these treatments. As we demonstrated in samples from well 12 and well 36, separating the groundwater from the sediment matrix altered the biodegradation potential of the community (Fig. 1 and Fig. 2). A nutrient amendment was required to restore the activity. Previous investigations have demonstrated that nutrient additions do not necessarily lead to drastic alteration of community function (Jones and Alexander 1988, Röling et al 2002). Moreover, the sequences recovered from the SIP experiments represented γ-and β-proteobacteria, which is a finding consistent with field investigations of benzene-degrading communities (Hendrickx et al 2006).

Subsequently, we presented convergent lines of cultivation-dependent and cultivation-independent inquiry to confirm that the members of γ-and β-proteobacteria are capable of degrading benzene and responsible for biodegradation in the microcosms. We coupled stable isotope probing (SIP) with cultivation and isolations to directly address potential methodological pitfalls in SIP that falsely implicate organism in SIP studies (Neufeld 2007). The isolation and pure-culture degradation experiments confirmed that the organisms, specifically *Variovorax* MAK3 and *Acidovorax* MAK5 are capable of the primary benzene degradation (Fig 2.6).

Cultivation approaches that supply BTEX compounds in vapor phase have been used in prior investigations, (Hendrickx et al 2006, Wang 2008). In Hendrickx et al (2006) they reported the cultivation *Acidovorax and Variovarox* spp from soil samples using BTEX mixtures. The results of our SIP and cultivation experiments reinforce a growing body of evidence that *Acidovorax* and *Variovorax* are active benzene degrading organisms in BTEX contaminated sites (Rooney-Varga et al 1993, Aburto et al 2009, Aburto and Peimbert 2011).

Variovorax spp in particular have been associated with soil habitats, and characterized as a metabolic diverse group of organisms with promising bioremediation and biotechnological applications (Han et al 2011, Miwa et al 2008, Boersma et al 2010). To the author's knowledge this is the first investigation demonstrating benzene degradation by *Variovorax spp* in pure culture, while simultaneously quantifying expression of the oxygenase gene involved in benzene attack (Fig 2.10).

We presented qPCR data that suggested the putative dioxygenase Vapar_5383 plays a role in benzene biodegradation by *Variovorax* MAK3. However, correlation does not prove the putative RHD is responsible for benzene degradation and quantitative PCR has inherent limitations particularly concerning the primer specificity and SYBR green assays. To improve the confidence that the primers were amplifying the target gene the amplicon was sequenced (Fig. 2.9) and the qPCR reaction was optimized to achieve a single peak in the disassociation curve. The transcript abundances from the pure culture experiment were consistent with the findings of Kong and Nakastu (2010), in comparison of kit based RNA-extraction methods for quantification of aromatic oxygenase genes.

Interestingly, the widely used Toluene/Benzene dioxygenase family primer sets: TOD (Baldwin et al 2003) and TODC1 (Hendrickx et al 2006) did not produce amplicons in the expected size range when applied to these *Variovorax* or *Acidovorax* isolates. Sequencing of the amplicons obtained using TOD and TODC1 primers yielded genes with no relation to aromatic dioxygenase genes. Witizig et al., (2006) completed a comparison of the toluene/biphenyl α-subunit-family gene sequences carried by *Variovorax paradoxus* EC4 to the classic dioxygenases in *Pseudomonas* ML2 (Benzene) and F1 (Toluene). Results of the sequence analyses revealed little similarity among dioxygenases (Witizig et al., 2006); this underscores the broad diversity of

oxygenase genes that occur in the environment and the potential limitations and biases of PCR-based inquiries (Yeates 2000, Gibson and Parales 2000). Despite high diversity among genes encoding enzymes involved in the attack of monoaromatic hydrocarbons, there seems to be a high degree of conservation in the biochemical pathways that allow destabilization of the aromatic ring. Supporting this notion, when we performed a metabolite extraction on cultures of *P. Putida* F1 and *Variovorax* MAK3 that were actively degrading benzene, we successfully recovered catechol from both cultures. Accompanying extraction and GC/MC analyses isolated only phenol from *Variovorax* MAK3, and not benzene cis-1,2-dihydrodiol. Benzene cis-1,2-dihydrodiol is a signature metabolite for dioxygenase attack of the benzene ring and this compound was successfully extracted from our benzene-degrading culture of *P. Putida* F1, suggesting that *Variovorax* MAK3 may attack the benzene ring via monooxygenation or that recover efficiency from the *Variovorax* culture was low. Further clarification on the precise nature of the enzymatic attack and pathway is required.

Introduction of the *Variovorax* MAK3 accelerated the loss of benzene in site-water microcosms, and the accelerated loss coincided with an increase in putative RHD abundance (2.11A and 2.11B). Surprisingly, the transcript abundances were significantly lower than those observed in the pure-culture experiments. This may be due to several contributing factors including, lower benzene concentrations in the incubations, increased competition, and/or slower cellular growth rate (Smith and Osborn 2008).

In the final panel (Fig. 2.11C) we normalized the expression of RHD with *Variovorax*-related16S rRNA transcripts to demonstrate the increased RHD expression as a function of the physiological status of the *Variovorax* community. We recognize that 16S rRNA is not a 'steady state' gene, and may exist in multiple copies, but here we chose to use 16S rRNA abundance to

examine the response of *Variovorax* populations native to the groundwater community to benzene addition. It is notable that *Variovorax*-related bacteria are native to the groundwater community and that specific RHD activity was boosted by the added benzene.

The specificity of the 16S rRNA-specific primer set developed by Bers et al 2011 is maximized by Hind III pretreatment on the template, which preferentially digests closely related *Acidovorax spp*. This step was not included in the procedures used here. Therefore we cannot exclude the possibility that our qPCR values may have included *Acidovorax spp.* However, the inclusion of *Acidovorax spp.* in our computed quotient (RDH#/16S rRNA#) would only diminish the values of quotients reported in Fig. 2.11C. Thus, our finding of increased RHD transcription in the presence of benzene may be quantitatively conservative. Also, we also cannot exclude the possibility the possibility that this RHD gene is present in other genera in site waters. The relative increase in expression we are attributing to *Variovorax* MAK3 could be a part of a larger community response.

Investigations into groundwater microbial communities using stable isotope probing face significant challenges given the inherent difficulty of accessing the habitat and controlling substrate dispersal in an open system. *In situ* approaches into groundwater systems have performed using Bio-Sep® beads carrying ^{13}C-labeled benzene (Geyer et al 2005, Kästner et al 2006, Stelzer et al 2005). Such assays can only be successful when the biodegrading populations colonize the Bio-Sep® beads and form a biofilm. These studies have largely focused on anaerobic benzene degradation in anoxic waters and relied on PLFA analysis for identification of the ^{13}C-labeled community members. SIP-based benzene biodegradation by microorganism from the coal-tar contaminated field site investigated in the present study have previously been reported by Liou et al. (2008)-- demonstrating that γ-and β-proteobacteria were active in both anaerobic

laboratory incubations and field SIP. However, there are many differences between both the physiological conditions and the inocula used by Liou et al. (2008; largely anaerobic sediments) and the inoculum and condition used in the present study; these difference make direct comparisons of the results of the two studies difficult.

The present study has demonstrated the ability of cultivation–dependent and cultivation-independent approaches to complement one another so as to produce new information about the identity of microorganisms carrying out key metabolic processes (in this case benzene biodegradation) in complex microbial communities.

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